# Long-chain alcohol production by yeasts

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# LONG-CHAIN ALCOHOL PRODUCTION BY YEASTS

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## SUMMARY

Several strains of yeast were analysed for long-chain alcohol content. *Candida albicans* contained the highest content with levels approaching 1.7 mg (g dry wt. organisms)$^{-1}$. Levels in *Candida sp. 107*, *C. tropicalis*, *C. maltosa*, *C.utilis* and *Pichia fermentans* were considerably lower. The content of long-chain alcohols in *C. albicans* grown anaerobically for 168 h in a medium containing 100 g glucose l$^{-1}$ increased considerably when the concentration of ammonium sulphate in the medium was lowered from 3.0 to 0.5 g l$^{-1}$. The increase was principally in the content of tetradecanol. The time-course of production indicated that long-chain alcohols are secondary metabolites and their production appears to be regulated by the carbon: nitrogen ratio of the medium. Maximum contents were achieved with a glucose concentration of 100 g l$^{-1}$ and an ammonium sulphate concentration of 0.5 g l$^{-1}$.

Differential centrifugation of spheroplast lysates of *C. albicans* indicated that long-chain alcohols were not located in the soluble or microsomal fractions. Purified cell-wall preparations from organisms grown for 168 h had very low contents of long-chain alcohols. When spheroplast lysates of *C. albicans* grown for 168 h were fractionated on sucrose-density gradients, the peak of long-chain alcohol content coincided with vanadate-sensitive, $Mg^{2+}$-requiring ATPase, a marker for plasma membrane. A smaller peak of long-chain alcohol content was found in a low-density fraction that is known to contain low-density vesicles that are involved in envelope growth.

Long-chain alcohol contents of organisms fell rapidly when cells from stationary-phase cultures were resuspended in fresh medium, the minimum content of approximately 0.5 mg (g dry wt. organisms)$^{-1}$ was reached at 16 h. The fall in tetradecanol content was proportionately higher than the fall in hexadecanol or octadecanol. Stationary-phase cells with high long-chain alcohol content showed a lag phase of approximately 12 h when resuspended in fresh medium whereas stationary-phase cells with low long-chain alcohol contents did not show this lag phase. Analysis of the medium showed that the long-chain alcohols were largely excreted upon re-inoculation.

The conversion of palmitoyl-CoA to 1-hexadecanol by cell-free extracts of *C. albicans* was found to require the presence of NADPH and a large excess of substrate. The enzyme activity had a pH optimum of 6.5 and the rate of hexadecanol formation was linear for 45 min. The reductase activity increased quickly as cultures entered the stationary phase of growth, but declined somewhat less rapidly thereafter. This decline in reductase activity slightly preceeded the fall in rate of production of hexadecanol.

Nitrosoguanidine was used to produce mutants of *Candida tropicalis* and a selection procedure based on cell buoyancy was used to select isolates with lower buoyancies than the wild-type. Many isolates were found to have defective triacylglycerol biosynthetic pathways, but mutants producing very high contents of long-chain alcohols were not found.

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# INTRODUCTION

## HISTORY OF YEAST LIPID RESEARCH

The earliest reference to microbial lipids was that of Nägeli and Loew published in 1878, reporting production of fat by a brewing yeast. Fractionation of yeast lipid was achieved by Hinsberg and Roos in 1903 and, since this time, the literature in this area has increased, although at a somewhat slower pace than that concerned with the chemistry and biochemistry of yeast carbohydrates and proteins. The reasons for the limited work on microbial lipids were largely due to difficulties in extraction and purification of these chemicals (Stodola *et al.*, 1967) and much of the initial work concentrated on screening yeasts as a possible commercial source of oil. In the 1940s and 1950s more sophisticated techniques of lipid analysis were introduced and research in the area expanded to include many aspects of lipid biochemistry (Rattray *et al.*, 1975). Up until this time, lipids had been considered purely as long-term storage compounds, but new analytical techniques such as thin-layer and gas-liquid chromatography allowed the identification of individual lipid components and permitted research into their role within the yeast cell. The significance of the quantitatively minor lipid components such as phospholipids, sphingolipids and lipoproteins was recognised and the introduction of radio-isotopes allowed the elucidation of many biochemical pathways concerned with lipid biosynthesis (e.g. Botham and Ratledge, 1979). Since this time a considerable amount of work has been carried out on membrane lipids, particularly on structure-function relationships of mitochondrial and plasma membrane lipids.

Recently there has been a resurgence of interest in the possibility of commercial production of microbial oil. Ratledge (1982) considered that there were two main reasons for the increased activity in this area. The first of these was that technology has been developed for large-scale cultivation of micro-organisms. This means that microbial production of single cell protein (SCP) is commercially viable and opens up the possibility that microbial production of yeast lipids could compete with plant products. The second reason was an increased volatility in the prices of both plant products and hydrocarbons, the major sources of most commercial lipids. Production of lipids via the fermentation of micro-organisms is regarded as a more reliable production method giving the stability of a monoculture and also the possibility of flexibility in terms of lipid composition (Moreton, 1988).

At the same time, work has been increasing in the area of microbial degradation of hydrocarbons (Ratledge, 1978). Originally research centred on the use of oil as a fermentation substrate, but more recently economic factors have encouraged research into the use of micro-organisms for synthesis and reclamation of hydrocarbons (Britton, 1984). Most recently, industrial research has centred on the possibility of using micro-organisms for production of high-value speciality lipids especially gamma-linolenic acid-rich oils and cocoa butter subsitutes (Slater, 1988) and functionally superior products such as ergocalciferol, an analogue of cholecalciferol, vitamin $D_3$ (Sekula,1988). Knowledge gained from microbial lipid research is the basis from which biotechnological opportunities can be explored and provides information allowing improvements in lipid production through metabolic or genetic manipulations of micro-organisms.

## MAJOR LIPID CLASSES

### Fatty-Acyl Residues

Due to the toxicity of free fatty acids (Hunkovà and Fencl, 1977) yeast cells do not contain significant amounts of these compounds. Large amounts (up to 20% of the total lipid) have been reported but this is likely to been an artefact due to lipid degradation during the extraction process (Ratledge and Evans, 1988). There have been reports of up to 5% fatty acids in the free form in *Saccharomyces cerevisiae* (Castelli *et al.*,1969; Hunter and Rose, 1972), *Lipomyces starkeyi* (Suzuki and Hasegawa, 1974a) and other yeasts, but fatty acids are normally found as esters of glycerol or sterols.

The fatty-acyl composition of yeasts is very variable and is affected by a variety of factors including nutrient concentration, pH value, temperature and oxygen tension. Cottrell *et al.* (1986) grew 18 strains of *Sacch. cerevisiae* on a glucose/nitrogen base medium and found great variation in all of the major fatty-acyl residues. A total of 33 fatty-acyl residues, ranging from $C_8$ to $C_{26}$ including isoprenoid type acids, were detected in *Sacch. cerevisiae* by Baraud *et al.*(1970).

Ratledge and Evans (1988) compiled a list of the relative percentage (w/w) of the major fatty-acyl residues from 14:0 to 18:3 chain length of a wide range of yeasts. The general conclusion that can be drawn from this compilation is that even closely related species show a preponderance of straight-chain $C_{16}$ to $C_{18}$ acids which may be saturated or unsaturated.

Polyunsaturated fatty acids are not found in *Sacch. cerevisiae* due to the absence of the appropriate $\Delta^{12}$ desaturase, but linoleic ($C_{18:2}$ , $\Delta^9$, $\Delta^{12}$) and linolenic ($C_{18:3}$, $\Delta^9$, $\Delta^{12}$, $\Delta^{15}$) residues are found in many other yeast strains, associated

with phospholipids and triacylglycerols (Ratledge and Evans, 1988). Shorter and longer chain residues tend to be found only occasionally and in small proportions. Odd chain-length residues ($C_{15:0}$, $C_{15:1}$, $C_{17:0}$ and $C_{17:1}$) are also usually in trace amounts when found in yeasts, except where the growth substrate is an odd-numbered alkane (Thorpe and Ratledge, 1972) or odd-chain length fatty acid (Bell, 1973). There are a few reports of branched chain fatty-acyl residues in yeasts (Kates and Baxter, 1962), but these are also only found in very low proportions.

Dicarboxylic fatty acids are produced by a number of yeasts after growth on alkanes (Hill *et al.*, 1986) and these may reach proportions that may be commercially useful. There have been a number of reports in the literature of mutants of *Candida tropicalis* and *C. maltosa* which have been developed to produce dicarboxylic acids from hydrocarbons in quantities up to 64 g $l^{-1}$. Various patents have been taken out to cover production of these fatty-acyl residues (Buhler and Schindler, 1984; Fukui and Tanaka, 1980, 1981; Rehm and Reiff, 1981).

## Sterols

Sterols are steroid alcohols, derivatives of the saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene. The hydroxyl group at C-3 represents the polar moiety of the molecule and the branched aliphatic side chain of eight to ten carbons at C-17 and the steroid skeleton constitutes the hydrophobic part of the molecule.

Sterols occur both free and esterified with long-chain fatty acids. Free sterols are associated with membrane functions whereas esters are storage compounds or biosynthetic intermediates.

The sterol content of yeasts generally ranges from 0.03 to 4.6% of the cell dry wt. and may account for less than 1% to 10% of total cell lipid. Sterols are found at particularly high levels in *Saccharomyces* species with ergosterol being the major component (Dulaney *et al.*, 1954). This is also the major sterol in *Kluyveromyces fragilis* and *C. albicans* (Rattray *et al.*, 1975). The next most abundant sterol is a precursor of ergosterol, 24(28)-dehydroergosterol which was found to be the predominant sterol in *Sacch. cerevisiae* NCYC 366 by Longley *et al.* (1968). Hunter and Rose (1972) used the same strain and found ergosterol and 24(28)-dehydroergosterol in equal proportions. Zymosterol has been detected in yeasts (Hossack *et al.*, 1977), as have a number of minor sterols including lanosterol and episterol.

The sterol composition is a key factor in the resistance of yeasts to polyene macrolide antibiotics and this has been used as an experimental system for studying the function of sterol in membranes (Hamilton-Miller, 1974).

**Acylglycerols**

Acylgylcerols are fatty-acyl esters of the trihydric alcohol glycerol. Mono-, di- and triacylglycerols have all been reported in yeasts. Triacylglycerols form a major storage component whereas diacylglycerols are biosynthetic intermediates. Monoacylglycerols are not storage lipids or intermediates in the biosynthesis of any other lipid and detection of both monoacylglycerols and high proportions of diacylglycerols has been attributed to lipolysis during lipid extraction either

through the action of lipases on triacylglycerols (Nurminen and Suomalainen, 1970) or phospholipases on phospholipids (Harrison and Trevelyan,1963).

A study of *L. starkeyi* by Suzuki and Hasegawa (1974a, b) and of various yeasts by Thorpe and Ratledge (1972) showed the *sn*-2 position on the glycerol molecule was invariably occupied by the unsaturated residues $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$. Thorpe and Ratledge (1972) also showed that about 50% of total triacylglycerol fatty-acyl residues in *Candida* sp. 107 and *Rhodotorula graminis* were unsaturated resulting in over 50% of the triacylglycerols being of the 1, 3-disaturated, 2-mono-unsaturated type. Growth of *Candida* sp107 and *C. tropicalis* on *n*-alkanes ranging from $C_{12}$ to $C_{16}$ in chain length caused the fatty acyl composition to vary according to the chain length of the substrate. Haley and Jack (1974) and Hammond *et al.* (1981) showed the *sn*-1 and *sn*-3 positions were occupied by different fatty-acyl residues in *Sacch. cerevisiae* and there was no evidence for a random distribution. The *sn*-1 position was shown to have a preponderance of unsaturated residues (approximately 85%) whereas $C_{16:0}$ and $C_{18:0}$ residues comprised 38% of the total substituents at the *sn*-3 position.

**Glycerophospholipids**

Glycerophospholipid is the general term applied to lipids containing a phospho-diester linkage as a mono- or di-ester, in which a hydrophilic head group is linked via a glycerol residue to a hydrophobic tail consisting of two long-chain fatty-acyl residues esterified to hydroxyl groups of the glycerol moiety.

Most yeasts have a phospholipid content of 3.0 to 7.0% of the cell dry wt. although higher contents have been obtained in yeasts grown on alkanes. A hydrocarbon substrate encourages development of internal membranes (Ludvik *et*

*al.*, 1968). These morphological changes are accompanied by an increase in phospholipid content needed to maintain membrane integrity (Thorpe and Ratledge, 1972).

A number of workers have reported the phospholipid composition of yeasts and it has generally been found that phosphatidylcholine is the major component, representing 25 to 55% of the total, with phosphatidyletholamine the next most abundant glycerophospholipid, representing 20 to 35% of the total. Phosphatidylinositol is usually present in the range of 7 to 21% and phosphatidylserine in a range of 4 to 19% (Letters, 1968; Kaneko *et al.*, 1976; Weete, 1980).

Most phospholipids are characterised by a high proportion of unsaturated fatty-acyl residues. This is probably a reflection of the functional role of phospholipids in membrane structures providing a requirement for fluidity rather than a crystalline structure (Longley *et al.*, 1968).

**Sphingolipids**

Sphingolipids are hydroxylated fatty-acid esters of long-chain amino-alcohols (sphingosines) and are found in most yeasts although the usual content is less than 0.5% of the cell dry wt. (Ratledge and Evans, 1988). These lipids do not occur in the free form but as an moiety of several yeast lipids ranging from simple cerebrins to complex glyco- and inositol phosphoryl sphingolipids (Rattray, 1975). Although the range of sphingolipids in yeasts is large and varied, the functions of the lipids are incompletely understood, although their frequent isolation from the cell envelope indicates a role in membrane integrity (Weete, 1980).

## Glycolipids

This term covers lipids consisting of long-chain fatty acids and alcohols covalently complexed with a carbohydrate. At least four acyl-glucose derivatives have been detected by Brennen *et al.* (1970) and these were suggested to be involved in storage or transport of glucose in yeast.

*Rhodotorula* spp. have been found to produce acylated derivatives of the polyols mannitol, arabitol and xylitol as extracellular lipids (Tulloch and Spencer, 1964). Other extracellular lipids which may have some commercial potential are the sophorose-containing lipids produced by *C. bombicola* and *C. bogoriensis*. These glycolipids consist of sophorose attached via a glycosidic link to a hydroxy fatty acid (Ratledge and Evans, 1988). If both a carbohydrate (glucose) and a source of acyl groups (sunflower oil) are available during growth the yield of sophorolipids may reach 70 g $l^{-1}$ (Cooper and Paddock, 1984).

## Hydrocarbons

Straight- and branched-chain alkanes with chain lengths from $C_{10}$ to $C_{31}$ were detected in *Sacch. oviformis* and *Sacch. ludwigii* (Baraud *et al.*, 1967) and Rattray *et al.* (1975) confirmed the presence of squalene in *Sacch. cerevisiae* by gas chromatography-mass spectrometry. Squalene is thought to affect membrane permeability through its influence on lipid molecule spacing.

### Other Lipids

White *et al.* (1987 and 1988) surveyed 14 strains of yeasts and reported the presence of fatty alcohols in a number of these. Amounts were small, averaging about 20 μg (g cell dry wt.)$^{-1}$, although larger amounts were found in *C. albicans*.

Various diol lipids have been isolated by Bergelson *et al.* (1966) from a *Lipomyces* sp. and Batrakov *et al.* (1974) reported both diacylesters and plasmalogen diol lipids in *Crytococcus laurentii, C. tropicalis* and *L. starkeyi*. A diol phospholipid was also isolated from *L. starkeyi* by Suzuki and Hagawa (1974c).

## CELLULAR DISTRIBUTION OF LIPIDS

### Cell Wall

Cell-wall preparations are obtained after rupture of the cell and removal of the internal components and plasma membrane by numerous washing steps. There remains, however, a distinct possibility that any cell-wall preparation may be contaminated by plasma membrane.

The lipid content of yeast walls is usually reported as a fairly low value, usually between 2 and 10% of the cell wall dry wt. In *Sacch. cerevisiae* the fatty-acyl composition of the wall lipid has been noted to be similar to that of the plasma membrane (Nurminen and Suomalainen, 1971). Domer and Hamilton (1971) maintained that the cell wall had a similar lipid composition to the cell sap in *Blastomyces dermatitidis* and *Histoplasma capsulatum* and that these yeast-like organisms had significant quantities of free and esterified sterol in the cell wall.

Sterols were also found in the cell wall of *C. albicans* by Bianchi (1967) but the walls of *Sacch. cerevisiae* have been found to be deficient in sterol, although the mannan component possesses a general capacity to bind sterols *in vitro* (Baraud *et al.*, 1970; Nurminen and Suomalainen, 1971).

**Plasma Membrane**

Two main strategies have been employed for the isolation of yeast plasma membranes. The first of these involves submitting yeast populations to solid-shear or liquid-shear stresses, which rupture the cell wall. The cell extract is then subjected to differential or density-gradient centrifugation. The second method involves enzymic removal of the yeast cell wall followed by lysis of the osmotically sensitive spheroplasts and purification procedures. Fuhrmann *et al.* (1976) used an alternative approach exploiting the differences in the surface charge densities of mitochondria and plasma membranes.

The composition of plasma membranes varies according to their source, but in general they contain approximately 40% lipid and 60% protein with some carbohydrate covalently linked to lipid or protein (Harrison and Lunt, 1980). It is generally agreed that yeast plasma membranes contain only amphipathic lipids, mainly glycerophospholipids and sterols. Triacylglycerols and sterol esters have been detected in plasma membrane preparations by some workers, but this is largely thought to be due to contamination by low-density vesicles (Henschke and Rose, 1990).

Phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol are the main glycerophospholipids of yeast plasma membranes, representing 70 to 85% of the total (Rank and Robertson, 1983). The glycerophospholipid component is

found to be essential for various structural roles and the intramembranous distribution of phospholipids is thought to be important for the control and integration of vectorial processes (Prasad, 1985).

Ergosterol is the major sterol isolated from yeast plasma membranes (Marriott, 1975) while 24(28)-dehydroergosterol, the precursor of ergosterol has also been found in the plasma membranes of *Sacch. cerevisiae* (Longley *et al.*, 1968). Studies with sterol auxotrophs have revealed the multiple functions of sterols within membranes. Sterols are thought to be essential for the maintenance of membrane fluidity and this requirement is met by a number of different sterols and stanols (Prasad, 1985).

The yeast plasma membrane is thought by most workers to resemble the fluid-mosaic matrix proposed by Singer and Nicolson (1972). This model envisages a liquid-crystalline phospholipid bilayer into which amphipathic, globular proteins are embedded. In addition, yeast plasma membranes also contain sterols and have a larger amount of protein than that proposed by the model. Membrane fluidity is brought about through the lateral mobility of lipids in the bilayer and interactions between phospholipids on a molecular level. The fluid-mosaic model postulates that all lipids are above their transition temperature. At this temperature a sharp rise in heat absorption occurs and the mobility of the hydrocarbon chain increases.

A limited amount of work has been published on the lipid composition of yeast plasma membranes, but there are considerable discrepancies over the relative contribution of various lipids to the overall composition of the membrane. Kramer *et al.*(1978) reported a phospholipid content of plasma membrane from *Sacch. cerevisiae* of 5 to 6% of the total plasma membrane lipid and Schneider *et al* (1978) reported a phospholipid content of *C. tropicalis* plasma membranes of less than 2%. By contrast Kaneko *et al.* (1976) found a phospholipid content of

over 50% and Nurminen *et al.* (1976) reported that over 80% of the total cellular phospholipid and sterol banded with the plasma membrane fraction on a density gradient. These latter two proportions are thought to be more realistic values (Prasad, 1985).

There are some data to suggest that the degree of unsaturation in yeast plasma membrane phospholipids can vary and this is found to influence the fluidity of the membrane. Anaerobically-grown *Sacch. cerevisiae* has a fairly non-specific requirement for a sterol and an unsaturated fatty acid, thus it is possible to grow this yeast under anaerobic conditions such that cells are enriched with particular sterol and fatty-acyl residues (Rose, 1977). Exceptionally high unsaturation values were detected in phospholipids from yeasts grown in the presence of multiply unsaturated acids and harvested in the mid-exponential phase of growth (Watson and Rose, 1980). The high degree of fluidity inferred from this level of unsaturation did not appear to affect basic growth parameters, but there was some evidence for instability in the plasma membranes of these organisms. Recent work by Pilkington and Rose (1989) supports the view that there is an upper limit to which the phospholipids of *Sacch. cerevisiae* can be enriched with unsaturated residues.

## Vesicles

*Saccharomyces cerevisiae* was reported to contain one or more large vacuoles and a number of small vesicles by Guilliermond *et al.* (1933). These vesicles have come to be known as spherosomes or, as they stain with lipid soluble dyes, lipid

granules and were thought to contain neutral lipids. Since these early reports, there have been frequent studies based on these organelles with confirmation of the presence of lipid.

Sentandreu and Northcote (1969) and Wiemken *et al.* (1970) showed that the vacuole fragments during the cell cycle to form vesicles which suggests that these vesicles are concerned with envelope growth. Clausen *et al.* (1974) isolated lipid particles from baker's yeast and found these to be rich in triacylglycerols and esterified sterols. Vacuoles and small vesicles were fractionated by rate centrifugation through a Ficoll gradient by Cartledge *et al.* (1977) and the lipid composition of these organelles reported by these workers differed in some respects from that reported by Clausen *et al.* (1974). The content of acylglycerols was found to be high, but the content of sterol esters was low and the vesicles were found to contain a substantial proportion of phospholipid (19.8%). These disparities may be due to the widely different methods used to obtain the vesicles.

Uzuka *et al.* (1975) isolated oil globules from *L. starkeyi* and found them to contain triacylglycerol as a major component and phosphatidylethanolamine as a minor lipid. Other phospholipids were important constituents in whole cells, but were not found to any great extent in the oil globules. The lipid-containing organelles were found in this yeast in both exponentially growing cells and cells in the stationary-phase of growth. These globules are thought to be similar and probably identical to the vesicles found in *Sacch. cerevisiae.*

Lipid vesicles were also isolated from bakers' yeast by Schaffner and Matile (1981) who found the major lipids to be sterol esters, triacylglycerols, sterols and phospholipids. These workers also suggested that vesicles represent a storage compartment of lipids that are eventually transformed into membrane lipids.

## Other Subcellular Organelles

Mitochondrial membranes have been obtained through differential centrifugation techniques and separated from other membranes using density gradients (Paltauf and Schatz, 1969) or differences in surface charge (Fuhrmann, 1976). Inner and outer mitochondrial membranes have been isolated by a number of different methods exploiting the physical differences between these two membranes (Bottema and Parks, 1980; Buttriss and Diplock, 1988).

There are relatively few reports on the lipid composition of mitochondrial membranes. Jakovic *et al.*(1971) found that 90% of diphosphatidylglycerol was found in the mitochondrial membranes of wild-type and petite mutant strains of *Sacch. cerevisiae.* This content varied with growth conditions and was thought to be a good indicator of the state of development of mitochondrial membranes.

Other principal phospholipids found in mitochondrial membranes are phosphatidylcholine and phosphatidyletholamine. The principal fatty-acyl residues found in aerobically grown *Sacch. cerevisiae* are oleic and palmitoleic acids and these are found in approximately equal proportions (Paltauf and Schatz, 1969).

Promitochondrial lipids were studied by Paltauf and Schatz (1969) and were found to contain a lower level of ergosterol and a simple and unusual fatty acid composition. Cells grown under anaerobic conditions with yeast extract, but no fatty acid or sterol supplement, were characterized by very low unsaturation of promitochondrial lipids and a high content of short-chain saturated fatty-acyl residues. These promitochondria retain the ability to support oligomycin-sensitive ATPase activity, but are unable to carry out respiratory functions

(Paltauf and Schatz, 1969). The high levels of saturated, short-chain fatty-acyl residues of promitochondria are found to be rapidly replaced by residues of oleic and palmitoleic acids after aerobic induction of mitochondria over a period of 30 to 120 minutes. This is accompanied by a marked increase in mitochondrial phospholipid and the development of mitochondrial function.

In a study by Bottema and Parks (1980) of mitochondrial membranes of *Sacch. cerevisiae* it was found that there was no difference in the phospholipid/sterol ratio in the inner and outer membranes. This contrasts with work in other tissues, for instance mammalian heart (Comte *et al.*, 1976) and *Neurospora crassa* (Hallermayer and Neupert, 1974) where sterols were found only in the outer mitochondrial membrane. Sterols provide both fluidity and rigidity to membranes and their presence is thought to be necessary in the inner mitochondrial membrane to ensure that enzymes have a suitable environment in which to function.

A number of other organelles including nuclei, vacuoles and endoplasmic reticulum have also been isolated by ultracentrifugation techniques, but information on the lipid composition of these structures is incomplete.

## FACTORS AFFECTING YEAST LIPID COMPOSITION

There are many factors that can lead to variation in the lipid content and composition of yeasts. Variations occur between different strains and there are also numerous cultural effects that exert an influence on lipid content and composition.

In considering total lipid contents two distinct groups of yeasts are recognised: i) those yeasts that contain up to 20% lipid and ii) those yeasts that have lipid

contents above 25% of the cell dry wt. (Rattray, 1984). Yeasts with high lipid producing capabilities are termed oleaginous while those producing lower levels of lipid are termed non-oleaginous. There are, of course, a large number of yeasts that produce lipid in the borderline region of 20 to 25% and their classification is largely arbitrary. In addition to this, there is the strong influence exerted by environmental factors such that an oleaginous yeast may be grown to contain less than 10% lipid. A more accurate classification of oleaginicity is the demonstration of the presence of the enzyme ATP:citrate lyase (Botham and Ratledge, 1979), but it is not always possible to analyse biochemically every potential oleaginous organism.

The pattern of lipid accumulation in batch culture in yeast is essentially a two-phase process. The first stage is the proliferation of cells at the maximum rate until a nutrient other than carbon becomes exhausted. In certain organisms the carbon source continues to be consumed and, although the growth rate slows, lipid continues to accumulate at the same rate (Kessel, 1968).

## Growth Rate

There are a variety of factors which affect the growth rate of yeast and these are a major determinant of cellular lipid content. The acylglycerol content of *Sacch. cerevisiae* has been examined during cell growth by Taylor and Parks (1979). These workers found that cells maintained a constant amount of phospholipid and diacylglycerol throughout growth. During early exponential phase of growth there was a decrease in triacylglycerol content which increased rapidly when the culture entered the stationary phase of growth. It was suggested that triacylglycerol was utilized for phospholipid synthesis during exponential growth through a diacylglycerol intermediate.

In batch cultures as the growth rate changes throughout the fermentation, the composition of the medium is modified by successive generations of cells. Thus there are changes in pH value, oxygen tension, temperature and nutrient concentration. Changes in growth rate can greatly affect lipid content as has been shown with chemostat cultures (Babij *et al.*, 1969; Brown and Johnson, 1970). Thus, when changes in environmental factors are being studied, the change in growth rate should always be taken into account.

## Sporulation

In *Sacch. cerevisiae* the process of sporulation is accompanied by an increase in lipid content (Chassang *et al.*, 1972 and Illingworth *et al.*, 1973). Sterol may make up to 81% of the total lipid content and it was ascribed a special role in the induction of sporulation by Chassang *et al.* (1972). In the initial phase of sporulation, there is formation of phospholipid and triacylglycerol, co-inciding with membrane development. In the second phase, there is synthesis of neutral lipids and abundant lipid granules are apparent in the ascospores, but these disappear when the cell returns to the vegative phase.

## pH Value

Using growth media with a pH value ranging from 3.0 to 7.5, only slight variations in lipid content have been observed in a variety of yeasts, including *Candida* sp. 107 (Hall and Ratledge, 1977) and *Rh. gracilis* (Kessel, 1968). Some

lipolysis of triacylglycerol appears to occur in *C. lipolytica* (Dyatlovitskaya *et al.*, 1969) and *Rh. glutinis* (Zalashko, 1974) grown at pH values outside the optimum range for growth.

## Temperature

Growth temperature has an effect on the total lipid content of yeasts. In general, as the temperature is lowered the lipid content increases. Kates and Baxter (1962) observed an increase in lipid content of *C. lipolytica* of 6.6 to 8.5% when the temperature was dropped from 25 to 10°C. *Cryptococcus albidus* (Hansson and Dostalek, 1986a) showed maximum lipid productivity at 20°C compared to 25°C and 30°C. An increase from 12.5 to 14.4% was observed when *Sacch. cerevisiae* was grown at 15°C rather than 30°C (Hunter and Rose, 1972). This increase was largely in triacylglycerol and phospholipid and because the culture was grown in a chemostat was thought to be a true temperature effect. *Candida* sp. 107 showed higher lipid production at 30°C than at 19°C or 33°C (Hall and Ratledge, 1977).

The degree of unsaturation of fatty-acyl residues has been shown to be affected by temperature in some yeasts (Brown and Rose, 1969; McMurrough and Rose, 1973) although the effect is not universal (Boulton and Ratledge, 1984). The changes which do occur in degree of unsaturation or chain-length have the effect of increasing the fluidity of the membrane lipids at lower temperatures and have been attributed to the activity of various enzymes including desaturases, acetyl-CoA carboxylase and fatty-acid synthetase (Ferrante *et al.*, 1983; Fulco, 1974; Hori, 1987).

# Nutrients

## Inositol

A deficiency of this vitamin has been observed to result in an increase in lipid content, especially triacylglycerol, in *Sacch. carlbergensis* (Johnston and Paltauf, 1970). These workers considered inositol deficiency to cause the fatty-acid synthetase system to be more active and this leads to an over-production of fatty-acyl residues and hence a higher triacylglycerol content. In addition to this, a deficiency of inositol in the medium leads to a decrease in phosphatidylinositol content (Henry, 1982), although other phospholipids continue to be made. A complete absence of this vitamin from the medium leads to inositolless death through disruption to membrane structure/function (Henry *et al.*, 1977).

## Pantothenic acid

Rattray *et al.*(1975) reviewed the effect of this vitamin on lipid biosynthesis. The absence of this growth factor from the culture medium caused a decrease in the total lipid content of *Sacch. cerevisiae* and *Hanseniasporo valbyensis*. Various changes in unsaturated fatty-acyl proportions content were also reported. Impaired mitochondrial development is thought to be associated with the disturbances in lipid content.

**Thiamin**

The absence of thiamin from a culture medium leads to a decrease in total lipid and a decrease in $C_{16:1}$ residues in *H. valbyensis*. Absence of thiamin is also found to produce lower proportions of unsaturated fatty acids in *Sacch. carlsbergensis* (Nishikawa *et al.*, 1974).

**Biotin**

Biotin deficiency has a wide range of effects including growth impairment. Suomalainen and Keranen (1968) found that lack of biotin lowered levels of $C_{16}$ and $C_{18}$ acids, but the addition of unsaturated acids to the growth medium permitted normal growth.

**Phosphate**

Phosphate limitation is thought to encourage lipid accumulation in some yeasts, although the effect may not be as wide-spread as that of nitrogen limitation (Ratledge, 1982). Johnson *et al.* (1973) found that phosphate limitation caused increased lipid content and an altered fatty-acyl profile in *C. utilis* and an increased triacylglycerol content in *Sacch. cerevisiae*. Gill *et al.*(1977) found an increase in lipid accumulation in *Candida* sp. 107 under phosphate-limited conditions, although this was not as high as under nitrogen-limited conditions. Maximum lipid contents (44% of yeast biomass) were achieved with dual limitation of both nitrogen and phosphate. No decrease in phospholipid content was observed under these conditions, although Gill *et al.* (1977) reported a decrease in sterol ester content from 5% to 2.5%.

## Nitrogen

Yeasts often accumulate lipids at a high rate when the nitrogen source is limited in the medium (Evans and Ratledge, 1983; Gill *et al.*, 1977; Hall and Ratledge, 1977). This is not due to depletion of the nitrogen source inducing a higher rate of synthesis, but rather that synthesis of proteins, RNA and DNA ceases and the excess carbon continues to be consumed and is diverted into lipid synthesis (Ratledge, 1982). In some yeasts, this leads to extensive lipid deposits within the cell, often as discrete droplets of lipid (Ratledge and Evans, 1988; Starkey, 1945; Uzuka, 1975).

Botham and Ratledge (1979) suggested a biochemical explanation for lipid accumulation in the oleaginous yeast, *Candida* sp. 107, under conditions of nitrogen-deficiency. Under conditions where the nitrogen source is limited, *Candida* sp. 107 builds up a supply of ATP leading to a depletion of AMP in the cell. The enzyme NADH-dependant isocitrate dehydrogenase is found to require allosteric activation by AMP in *Candida* sp. 107. Thus, in nitrogen-limited cultures this enzyme will be inactive and this will lead to a build-up of isocitrate. Aconitase is unaffected by changes in energy charge and this enzyme will allow equilibration of isocitrate and citrate which will be transported out of the mitochondrion. In *Candida* sp. 107, ATP:citrate lyase will be active under these conditions and this will cleave citrate to oxaloacetate and acetyl-CoA. This latter compound becomes available for fatty acid biosynthesis and lipid accumulation. Similar characteristics were found by Botham and Ratledge in oleaginous strains of *Rh. glutinis* and *Mucor cirinelloides*, but the equivalent enzymes in *C.utilis*, a non-oleaginous yeast, were found to be unaffected by nitrogen depletion.

Bati *et al.* (1984) also found that nitrogen concentration was an important factor in lipid accumulation in *C. lipolytica* grown on corn oil as a sole carbon source. In this case, it was found that a nitrogen-source concentration of 124 mg $l^{-1}$ was the optimum and both higher and lower concentrations depressed biomass production and restricted lipid accumulation in the yeast.

The carbon:nitrogen (C:N) ratio has been found to have an effect on lipid production in a number of yeasts. Turcotte and Kosaric (1989) found an optimum initial molar C:N ratio of 77 for maximum lipid production by *Rhodosporidium toruloides*. A mathematical model has recently been developed which brings out the critical nature of the effect of the C:N ratio in the medium on yeast lipid production (Sattur and Karanth, 1989a, b, c). This model provides a useful theoretical prediction of biomass and lipid production, although there is not total agreement with experimental observations due to incomplete substrate utilization.

Studies on *Rhodosp. toruloides* by Evans and Ratledge (1984) showed a higher fat co-efficient when grown in a medium with only an organic nitrogen source. It has also been reported that inorganic nitrogen sources in media need to be supplemented by an organic nitrogen source for oleaginicity (Weete, 1980). However, Moreton (1988) states that there is no firm evidence for a preference for a particular nitrogen source, and postulates that the increased buffering capacity of organic nitrogen sources favours lipid production.

Some work has also been carried out on the oleaginous yeast *Cryptococcus albidus* var *albidus* (Hansson and Dostalek, 1986 a, b; Krylova *et al.*, 1984; Pederson, 1961). This yeast appears to be able to accumulate lipid under nitrogen-limited and under excess-nitrogen conditions, although maximum production was observed when the nitrogen source was limited. High fat levels in

*Cr. albidus* seem to be constitutive although growth rate seems to be an important factor in lipid accumulation under nitrogen-limiting conditions in this yeast.

## Carbon Source

For a great many years it has been known that the type and concentration of the carbon source on which a yeast is grown has a wide-ranging effect upon the metabolism of the organism. Some of the first experiments were carried out by Pasteur and these were extended by Crabtree (Kappeli, 1986). As most yeasts can grow on glucose (Barnett, 1976) the metabolism of this carbon source has been studied most extensively. Most recent work has attempted to explain the metabolic effects exerted by glucose in biochemical terms (Fiechter *et al.*, 1981; Holzer, 1976; Kappeli and Fiechter, 1982; Kappeli *et al.*, 1985 a, b; Kappeli, 1986b).

Yeasts can be classified into three groups according to their glucose metabolism under carbon and oxygen limitations (Fiechter *et al.*, 1981; Kappeli, 1986b). The first of these are termed glucose-insensitive yeasts (e.g. *Trichosporon cutaneum*; Kappeli and Fiechter, 1982) in which exponential growth occurs until complete depletion of the limiting medium component. There is no ethanol formation even under oxygen limitation. The second type are the oxygen-sensitive yeasts (e.g. *C. tropicalis*; Kappeli, 1986b) which produce ethanol under oxygen limitation. The glucose-sensitive yeasts (e.g. *Sacch. cerevisiae*; Kappeli, 1986b) form the third group and these yeasts form ethanol even under aerobic conditions. This latter group of yeasts shows diauxic growth. In the first phase, biomass is formed and ethanol produced; in the second phase, when glucose is exhausted, ethanol serves as the carbon source. Glucose-sensitive yeasts have been extensively discussed by Kappeli (1986b) who asserts that cells exhibiting aerobic ethanol

formation have a branched system of glucose breakdown. If the terminal electron acceptor is oxygen, glucose is catabolized respiratively. If the terminal acceptor is acetaldehyde, glucose is catabolized fermentatively and this type of metabolism was termed respiro-fermentative by Käppeli. This is thought to be an overflow reaction at the level of pyruvate when respiration is saturated (Rieger *et al.*, 1983; Käppeli *et al.*, 1985a). Catabolite repression is a key characteristic of the metabolism of these yeasts (Holzer, 1976).

Brown and Johnson (1970) studied the effect of increasing glucose concentration in the incubation medium from 2 to 10 g $l^{-1}$ on the lipid content in *Sacch. cerevisiae.* The total lipid, sterol ester and phospholipid contents all decreased and there was a disproportionate decrease in unsaturated fatty acyl-residues. In addition to this there was a simultaneous decrease in mitochondrial lipid, but this may have been due to the state of anaerobiosis induced by high concentrations of glucose (Lowden *et al.*, 1972). Johnson *et al.* (1972) extended this study and found that increased glucose concentration led to decreased lipid content in *Sacch. carlsbergensis* and *Sacch. delbruekii* in addition to *Sacch. cerevisiae.* All of these yeasts are of the glucose-sensitive type and show aerobic ethanol formation.

The above study also included the glucose-insensitive yeasts *Sacch. fragilis*, *Schwanniomyces occidentalis* and *C. utilis*, and these yeasts were found to have an elevated lipid content when the glucose concentration was raised. This phenomenon had been noted earlier by Babij *et al.* (1969) in their work on the lipid composition of *C. utilis* and was repeated later in many other glucose-insensitive yeasts including *Candida* sp. 107 (Hall and Ratledge, 1977) and *Rh. glutinis* (Yoon and Rhee, 1983). Lipid accumulation could be elevated further if another nutrient, such as the source of nitrogen or phosphorus, was present in limiting concentrations. Thus it would seem that the regulatory effect of glucose

plays an important role in the extent of lipid accumulation in many yeasts with widely differing effect in glucose-sensitive and glucose-insensitive yeasts.

A variety of other carbon substrates can be metabolised by yeasts and variations in total lipid content have been recorded. *Rhodotorula gracilis* was grown on cellobiose, sucrose, glucose and xylose giving lipid contents of 39, 47, 57 and 62%, respectively (Yoon *et al.*, 1982). Hansson and Dostálek (1986a) used a variety of different carbon sources as substrates for *Cr. albidus*. Maximum lipid contents were obtained for glycerol (44%) and mannitol (43%) and minimum contents with xylose (33%) and lactose (26%).

Lactose, the major carbohydrate of whey, has also been studied as a potential substrate by Hammond's research group in the USA (reviewed by Ratledge and Evans, 1988). There seems to be a promising rate of conversion into lipid by *C. curvata*. *Lipomyces starkeyi* has been grown on starch with a lipid yield of 24% (Guerzoni *et al.*, 1985).

Hydrocarbons have also been extensively studied as an alternative carbon source for yeasts. The petroleum industry has an interest in the capacity of yeasts to metabolise hydrocarbons, not only in terms of alkane degradation, but also conversion into alternative products (Britton, 1984; Uemura *et al.*, 1988). Early research in this area concentrated on the use of microbes for waste disposal from refineries and the synthesis of industrially useful compounds, but more recently microbial recovery of hydrocarbons has been studied extensively. The possibility of producing single-cell protein through growth of yeast on hydrocarbons has also been investigated (Shennan and Levi, 1974).

Schauer and Kohler (1987) estimated that only about 20% of yeasts were capable of utilizing hydrocarbons as growth substrates. Tausson (1939) first reported the

assimilation of *n*-alkanes by members of the genera *Debaryomyces, Endomyces, Hansensula, Torulopsis* and *Monilia* (*Candida*). This capacity is not found in *Saccharomyces* species nor many other yeasts. The specific growth rate of yeasts may be increased (Ratledge, 1968) or decreased (Einsele *et al.*, 1972) when grown on hydrocarbons. There may also be an increase (Nyns *et al.*, 1968; Ratledge, 1968) or decrease (Thorpe and Ratledge, 1972) in quantities of total lipid and this is thought to be due to varying metabolic capacities of different strains, variations in environmental conditions or a combination of these effects (Rattray *et al.*, 1975).

Fatty acids are the major products of the metabolism of hydrocarbons, but small quantities of *n*-alkanes may also accumulate (Fukui and Tanaka, 1981). In many cases, the nature of the fatty-acyl component of the cellular lipid reflects the composition of the substrate (Ratledge, 1968). Alkanes of chain length less than $C_{13}$ are converted to various fatty acids by both chain elongation and *de novo* synthesis after *B*-oxidation of the substrate. Alkanes with longer chain lengths generally yield fatty acids of the equivalent chain length (Mishina *et al.*, 1973).

**Oxygen Tension**

The oxygen tension in a culture can have a pronounced effect on both total lipid levels and the composition of the lipid produced. Most studies on the effect of oxygen supply on lipid accumulation have been carried out on batch cultures where the growth rate changes and depends in aeration rates. More accurate studies involve the controlled environment of the chemostat (Prasad, 1985). Hall and Ratledge (1977) studied the effect of aeration rate on *Candida* sp 107 grown in continuous culture. The aeration rate was altered from 0.05 to 0.5 (v/v, air/medium) a minute and an increase from 10% lipid to 24% lipid was detected.

A further increase in aeration rate to 1.0 (v/v) a minute led to a decrease in total lipid. Choi *et al.* (1982) observed a similar pattern of lipid accumulation in *Rh. glutinis*. Brown and Rose (1969) studied the effect of oxygen availability on *C. utilis* and discovered that synthesis of polyunsaturated fatty-acyl residues depended on the oxygen tension. At high oxygen levels there was a high content of linolenoyl residues; as the oxygen tension dropped the degree of unsaturation decreased and $C_{16}$ residues accumulated at the expense of $C_{18}$ residues. Davies *et al.* (1990) found that, at oxygen uptake rates lower than 7 mmol $O_2$ $l^{-1}$ $h^{-1}$, the levels of total unsaturated fatty-acyl residues in *Apiotrichum curvarum* decreased.

*Saccharomyces cerevisiae* and other glucose-sensitive yeasts show an increase in total lipid content when grown under aerobic conditions (Brown and Johnson, 1970; Johnson and Brown, 1972). More specifically, there is an increase in phospholipid and sterol contents with the acylglycerol fraction being somewhat variable (Jollow *et al.*, 1968; Kovac *et al.*, 1967). Ahvenainen (1982) studied the lipid composition of *Sacch. cerevisiae* grown aerobically and anaerobically and found aerobically-grown organisms contained 86% unsaturated fatty-acyl residues while unsaturated fatty-acyl residues constituted only 38% of the total in anaerobically-grown cells.

*Saccharomyces cerevisiae* grown under anaerobic conditions has a requirement for a sterol (Andreason and Stier, 1953) and an unsaturated fatty acid (Andreasen and Stier, 1954). The requirement for an unsaturated fatty acid seems to be fairly non-specific (Nes *et al.*, 1984), but a number of workers (Nes *et al.*, 1976 and 1978; Pinto and Nes, 1983) have found a variation in the ability of various sterols to support anaerobic growth.

## SECONDARY METABOLISM

Metabolism of certain micro-organisms and higher plants can be subdivided into two broad categories, namely primary and secondary metabolism. The criteria by which products are assigned primary or secondary status are various, but a primary metabolite tends to be a relatively simple chemical compound that is present in the organism throughout the life cycle and is generally necessary for growth of the organism. A secondary metabolite by contrast tends to be a complex chemical compound that is relatively free from turn-over and unnecessary for growth of the organism (Bennett, 1983). However, Campbell (1984) advocated a more fundamentally significant definition based on a distribution criterion such that primary metabolites are products of normal cellular metabolism that are widely distributed in nature whereas secondary metabolites are products of normal cellular metabolism that are more restricted in their distribution. This definition encompasses the idea that primary metabolism represents a general solution to a general biological problem, whereas secondary metabolism represents a specific solution to a general or a specific biological problem.

Observations on the synthesis of antibiotics during batch culture using complex media showed a biphasic pattern. The secondary products were not produced during the period of rapid growth, the trophophase, but at a later phase, the idiophase, after active growth had ceased (Bu'lock *et al.*, 1965). However, the trophophase and idiophase are not always separate in time and a secondary metabolite may be produced during the growth phase (Aharonowitz and Demain, 1980).

Secondary metabolites constitute a chemically diverse group, but they are all produced from a few key intermediates of primary metabolism and are generally

categorized according to the precursors from which they are synthesized (Bennett, 1983). Metabolites derived from components of sugar metabolism include glycosides and streptomycin antibiotics. Secondary metabolites synthesized from fatty acids include polyketides, terpenes and steroids. Secondary metabolites synthesized from amino acids in which the carbon skeleton is largely derived from the amino acid with the retention of the nitrogen include the ergot alkaloids, lysergic acid diethylamide and penicillins. There is also a large number of metabolites that derive carbon from more than a single source.

There has been a wide diversity of theories advanced to explain the significance of secondary metabolite production in micro-organisms. These various explanations reflect the diversity of secondary metabolites and are not necessarily mutually exclusive. The first theory put forward was that secondary metabolites are shunt products synthesized during the idiophase (Foster, 1949) and from this arose the postulation that secondary metabolism represents a detoxification process (Luckner, 1971). Secondary metabolism may have evolved as an alternative strategy to switching off metabolic pathways such that an accumulated pool of primary products may induce subsidiary pathways to form secondary metabolites (Malik, 1980). This may serve to maintain mechanisms essential for cell multiplication in operative order when cell multiplication is no longer possible (Bu'lock, 1961). Secondary metabolites may also confer survival advantages in nature, increasing the flexibility of cells to adapt to changing environments or eliminating possible microbial competitors. Some of these metabolites appear to have a negative impact in the producing organism when exposed to it during a rapid growth period and delaying synthesis until after growth may allow the organism to gain a selective advantage without the disadvantage of autotoxic effects (Drew and Wallis, 1983).

## OCCURRENCE OF LONG-CHAIN ALCOHOLS

Fatty alcohols are found in a wide range of living organisms in both free and esterified forms. They are commonly found in waxes as simple esters of long-chain fatty acids, but are also found as alkoxy lipids in fish liver oils. Alcohols are precursors of alkyl glycerol ethers which are components of some lipids in animal and plant tissues (Mahadevan, 1978). Most natural fatty alcohols are even-numbered primary alcohols, although several secondary alcohols have also been reported.

Marine wax esters are animal products distributed throughout a wide range of species and are especially abundant in organisms that experience periods of food shortage, often in polar and subpolar regions (Sargent, 1978). The long-chain alcohol moieties are invariably either saturated, especially 16:0 or monounsaturated, especially 18:1 ($\Delta^9$), 20:1 ($\Delta^9$) and 22:1 ($\Delta^9$). The fatty-acyl component is much more variable.

Terrestrial animal waxes are less common although they are present in significant amounts in sebum and beeswax. The alcohols in the beeswax of *Apis millifica* are mainly saturated, straight chains with 30 to 32 carbons (Holloway, 1969) whereas bumblebee wax from *Bombus rufocinctus* contains more branched chains of 4 to 6 carbons fewer than beeswax (Tulloch, 1971). The cuticular waxes of scale insects contain various amounts of free and esterified alcohols of chain lengths varying from 26 to 36 carbon atoms (Mahadevan, 1978).

Long-chain alcohols have also been extracted from normal and neoplastic mammalian tissues (Blank and Snyder, 1971; Takahashi and Schmid, 1970). They are present as wax esters in specialized glands (Snyder and Blank, 1969), but they occur more commonly as alkyl glycerol ethers in the mucosa of the small

intestine (Paltauf, 1971), mammalian brain and heart (Natarajan and Schmid, 1977) and at higher levels in transplantable neoplasms (Blank and Snyder, 1971). Long-chain alcohols are also precursors of mammalian phosphoglycerides (Wykle and Synder, 1976).

Plant waxes were reviewed by Kolattukudy (1970a) and found to consist mainly of wax esters, free fatty alcohols, free fatty acids and hydrocarbons. Plant wax esters usually consist of even-numbered alcohols in the range of $C_{12}$ to $C_{32}$; branched chains and double bonds are rare. The free fatty acids usually have a corresponding structure (Kolattukudy, 1970a). Jojoba seeds contain approximately 50% of the dry seed wt. as a liquid wax (Pollard *et al.*, 1979; Wu *et al.*, 1981). This wax acts as a major storage reserve and consists of wax esters with chain-lengths of 40-44 carbons.

There have been a number of reports of the existence of long-chain alcohols in bacteria, mainly in the form of bacterial wax esters. Russell's group found that the psychrophilic bacterium *Micrococcus cryophilus* contains fairly large amounts of wax ester, representing 14% of the total lipid (Lloyd and Russell, 1983 a, b; Russell and Volkman, 1980). The wax contained a predominance of 18:0 and 18:1 fatty acids and alcohols in a random combination. The chain length of the wax esters and the degree of unsaturation were found to be influenced by growth temperature. The authors suggested a role for the wax ester in maintaining membrane fluidity.

An extensive study of 45 strains of bacteria classified in the family *Neisseriaceae* (Bryn *et al.*, 1977) showed the presence of wax esters in some species of *Moraxella, Acinetobacter* and "false neisseriae". The chain lengths of the wax esters ranged from $C_{24}$ to $C_{42}$, with $C_{36}$ predominating and even-numbered esters constituting 70 to 100% of the total in all cases.

*Acinetobacter calcoaceticus* has been studied extensively in terms of wax accumulation (Fixter and Fewson, 1974; Fixter and McCormack, 1976; Fixter *et al.*, 1986; Gallagher, 1971). Wax esters were accumulated to the greatest extent by bacteria in stationary-phase, nitrogen-limited batch cultures and were shown to consist of $C_{14}$ to $C_{18}$ saturated or mono-unsaturated primary alcohols, esterified with $C_{14}$ to $C_{18}$ fatty acids. The wax ester was converted to water-soluble materials and carbon dioxide upon incubation of the bacterium in the absence of an energy source and it was proposed that wax ester served as an energy reserve.

Free long-chain alcohols have been found in the lipid component of *Escherichia coli* (Naccarato, 1972) and *Clostridium butyricum* (Day and Goldfine, 1978). In *E. coli* the major alcohols were established to be 1-tetradecanol (18%), 1-hexadecanol (28%), 1-octadecanol(14%) and 2-pentadecanol (27%). Anaerobically-grown cells showed a selective decrease in the content of secondary alcohols, suggesting separate metabolic pathways for the two types of alcohol in *E. coli* (Naccarato, 1972).

Reports of long-chain alcohols in yeast are rare. The accumulation of wax esters after growth on alkanes has been reported for *C. tropicalis* (Davidova *et al.*, 1978), *C. guilliermondii* (Muratov *et al.*, 1979) and *Rh. glutinis* (Zalashko and Salokhina, 1982). *Candida tropicalis* also accumulated wax esters when grown on glucose, but to a lesser extent than when grown on hydrocarbons (Davidova *et al.*, 1978). The most extensive study on long-chain fatty alcohols in yeast was by White *et al.* (1987 and 1988). These workers surveyed 14 strains of yeasts for long-chain alcohol production with glucose as a substrate. A number of these yeasts accumulated fatty alcohols, *C. albicans* proving to be the best producer in the survey. The alcohols produced were all primary, saturated alcohols of chain lengths $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$, and the time course of production suggested the

alcohols were secondary metabolites. The effect of glucose concentration was studied and it was found that fatty alcohol production was favoured by conditions of high concentrations of glucose and under oxygen limitation. Substitution of glucose in the medium with galactose decreased long-chain alcohol production and substitution with glycerol completely abolished it, suggesting involvement of carbon catabolite control.

## BIOSYNTHESIS OF LONG-CHAIN ALCOHOLS

It was suggested by Paltauf (1971) that there are three main pathways by which fatty alcohols are formed. Long-chain alcohols may be produced as intermediates during oxidation of hydrocarbons (Britton, 1984; Klug and Markovetz, 1971), by reduction of (activated) fatty acids (Naccarato *et al.*, 1972, 1974; Natarajan and Schmid, 1977; Wang *et al.*, 1972) or by reduction of aldehydes arising as cleavage products from sphingosine bases or alkoxy lipids.

The most common pathway of alkane degradation is oxidation at a terminal methyl group (Britton, 1984). Finnerty and Kallio (1964) observed that esters formed from alcohols and fatty acids were homologous to the original substrate indicating oxidation of the alkane to the primary alcohol. Since these original observations, formation of alcohols has been confirmed in many organisms and a transient accumulation of fatty alcohol has been observed in some cases (Ratledge, 1980; Suzuki and Ogawa, 1972).

Oxidation of *n*-alkanes to the corresponding *n*-alcohol is catalysed by a complex hydroxylase which may be linked to one of several different electron-carrier systems (Ratledge, 1978). The two most widely studied systems are linked either to cytochrome P-450 or to rubredoxin. The cytochrome P-450 system was first

studied in a *Corynebacterium* sp. by Cardini and Jurtshuk (1968 and 1970) and there are several lines of evidence favouring this mechanism in yeasts (Fukui and Tanaka, 1981). The enzyme is classed as a mixed-function oxidase and the system consists of cytochrome P-450 and NADPH cytochrome *c* reductase. Coon *et al.* (1969) reported the presence of this system in *C. tropicalis* and *C. guilliermondii* and its presence in *C. tropicalis* has been confirmed by Gallo *et al.* (1971 and 1973).

The other well-documented mono-oxygenase system is that in *Pseudomonas oleovorans* reported by Peterson and Coon (1968). This system involves rubredoxin, a NADH-rubredoxin reductase and a *w*-hydroxylase. This system is distinct from the cytochrome P-450 system, but a primary alcohol is the end-product of both.

Fatty alcohols are the initial product of both of these oxidations, but these are oxidised further to aldehydes and then fatty acids. The accepted mechanism for this next step has been dehydrogenation of the alcohol (Gallo *et al.*, 1973 and 1976; Tassin and Vandecasteele, 1972), but recently an alcohol oxidase has been proposed (Kemp *et al.*, 1988).

The amounts of fatty alcohols accumulating during alkane oxidation are low and values are rarely quoted. There have been a number of attempts at increasing the quantity by careful control of the oxidising conditions (Fredricks, 1967) or by producing mutants blocked at an appropriate point (e.g. Jenkins *et al.*, 1972). However, to date a successful process for production of a fatty alcohol from an alkane has not been developed and the current prohibitive cost of hydrocarbons make this unlikely.

The enzymic reduction of fatty acids and acyl-CoAs to long-chain alcohols has been noted in animals (Bishop and Hajra, 1978 and 1981; Bourre and Daudu, 1978), plants (Khan and Kolattukudy, 1975; Kolattukudy, 1971) and bacteria (Day and Goldfine, 1978; Naccarato *et al.*, 1974). White *et al.* (1988) supplemented cultures of *C. albicans* with odd-chain length fatty acids and observed formation of odd-chain length fatty alcohols. In unsupplemented cultures no odd-chain alcohols were detected, indicating that reduction of fatty acid was probably the pathway by which fatty alcohols are produced in this yeast.

In all systems studied, reduction of fatty acids appears to proceed via their activation to acylated derivatives, followed by reduction by NADPH or NADH (Riendeau and Meighen, 1985). In most cases the acylated compound is an acyl-CoA although an acyl-protein intermediate has been identified in *Photobacterium phosphoreum* (Riendeau *et al.*, 1982; Rodriguez *et al.*, 1983). Formation of acyl-CoA is catalyzed by acyl-CoA synthetase and this step is usually rapid and is not rate limiting. The acyl-CoA is reduced to an aldehyde by an acyl-CoA reductase and then further reduced to the corresponding alcohol by an aldehyde reductase. The rate of fatty acid reduction to alcohol is optimal near neutral pH values (Griffith *et al.*, 1981; Kolattukudy, 1970b; Thyagarajan *et al.*, 1979).

Synthesis of acyl-CoA from fatty acid is found to require coenzyme A, ATP and $Mg^{2+}$ (Kolattukudy, 1970b). Direct reduction of the carboxyl group of the fatty acid requires activation prior to reduction in order to be energetically feasible. Reduction of the acyl-CoA is found to require NADH or NADPH. The aldehyde intermediate has been identified in a number of cell-free systems, for instance *Mycobacterium tubercuolsis* (Wang *et al.*, 1972), *Cl. butyricum* (Day *et al.*, 1970), *Brassica oleracea* (Kolattukudy *et al.*, 1981) and bovine cardiac muscle (Johnson and Gilbertson, 1972), but is usually not found as a free intermediate. The cases where the aldehyde intermediate is found to accumulate involve soluble

enzyme preparations and in some cases the enzyme can be resolved into separate acyl-CoA reductases and aldehyde reductases (Day and Goldfine, 1978; Johnson and Gilbertson, 1972). In the majority of membrane systems studied, however, fatty aldehydes do not appear to be released as free intermediates and aldehyde-trapping agents have little effect on production of fatty alcohol (Bishop and Hajra, 1981). It is not yet clear whether the aldehydes are channelled between different enzyme systems or are products of a multifunctional enzyme complex. The study of membrane-bound reductases has been hindered by difficulties encountered in solubilizing the enzyme and limited success only has been achieved with *Euglena gracilis* (Khan and Koluttukudy, 1975).

Microsomal reductases show a high specificity for NADPH, with the exception of *E. gracilis* (Khan and Kolattukudy, 1973), whereas soluble systems are generally NADH specific. The soluble systems analysed to date are only capable of reducing the activated fatty acid whereas membrane-bound systems can reduce both the fatty acid and the acyl-CoA. This indicates that the fatty-acid activating system and the reductase are dissociated on preparation of the soluble system, or that the fatty acid activating system has a membrane location (Riendeau and Meighen, 1985).

To date, the only information available on long-chain alcohol biosynthesis in yeasts indicates that alcohols are produced via a reductive route. Preliminary studies by White *et al.* (1988) indicate that both microsomal and soluble fractions are capable of reducing palmitoyl-CoA to the corresponding primary alcohol.

## YEASTS AS A COMMERCIAL SOURCE OF LONG-CHAIN ALCOHOLS

Fatty alcohols are a valuable raw material of some importance to the chemical and detergent businesses. At present about £300 million worth of fatty alcohols are produced annually; the most important use is in the production of sodium alkyl sulphates, an important detergent ingredient. Non-ionic detergents are produced upon the reaction of long-chain alcohols with ethylene oxide on propylene oxide. In 1976 long-chain alcohol-derived surfactants had a world market of more than £0.5 billion and other new compounds such as surface-coating and waterproofing agents may increase this market. In addition to this, fatty alcohols are raw materials for various lubricants and cosmetics (Swern, 1982).

At present long-chain alcohols are produced either synthetically from petroleum products or from natural oils and fats by various hydrogenation processes. In 1983, 70% of the world production of fatty alcohols was from a petrochemical source (Buchold, 1983). The Nissan Chemical Oxo process produces alcohols in the $C_{12}$ to $C_{15}$ range and involves the reaction of an olefin with carbon monoxide and hydrogen. The reaction is carried out in one stage to produce alcohols, or the aldehyde may be isolated and reduced to the alcohol. The process requires a temperature of 75 to 200°C, pressure of 100 to 300 atmospheres and a cobalt catalyst. The Ziegler process is another important synthetic route producing alcohols in the range of $C_2$ to $C_{12}$ (Mahadevan, 1978).

Production of fatty alcohols from natural oils and fats such as coconut oil, palm oil and tallow is achieved with hydrogen at temperatures up to 350°C and pressures up to 200 atmospheres with a metal catalyst (Mahadevan, 1978). A plant in the Philippines designed to make fatty alcohols by direct hydrogenation of coconut oil uses a finely divided copper chromite catalyst and a temperature of 300°C (Buchold, 1983).

Neither the synthesis of fatty alcohols from petrochemicals nor the extraction from natural oils and fats are completely satisfactory processes. Crude oil is subject to rapidly escalating costs and the high temperatures and pressures involved in this synthetic process make production of fatty alcohols by this route very costly. The international prices of natural oils and fats have recently suffered from a certain degree of volatility (Moreton, 1988) and, despite the lifting of market restrictions in the European Economic Community in 1992, the demand for natural fats in European countries will be in excess to supply (Ratledge, 1982). In addition to this, raw materials from plant and animal sources generally originate in areas at risk from instability of both the political and natural environments. It is therefore of interest to the detergent and chemical businesses to explore the possibility of synthesising fatty alcohols from a microbial source, giving the stability of a monoculture. Rattray, in his Overview of Biotechnology in the Fats and Oils Industry (1984), believed the major impact of biotechnology to be on high-value, speciality products including cocoa butter substitutes, biosurfactants and waxes and the possibility exists of extending this to include fatty alcohols. The main advantages lie in the unique ability of micro-organisms to catalyse certain chemical transformations under mild reaction conditions. This can introduce a degree of flexibility into the product, in the case of fatty alcohols this is in terms of chain length and degree of saturation. The effects of three variables: solvents, feedstocks and temperature in oleochemical biosynthesis were reviewed by Neidleman and Geigert (1984) and the far reaching effects of these variables led these authors to predict a role for biotechnology within the oleochemical industry.

The object of this project was to study the production of long-chain alcohols by yeast. This study falls into three main areas, namely production, biosynthesis and subcellular location of fatty alcohols in yeasts.

# MATERIALS AND METHODS

## ORGANISMS

The yeasts used were *Candida albicans* NCYC 1467, *C. maltosa* Colworth Microbial Culture Collection (CMCC) 3152, *Pichia fermentans* NCYC 850, *C. utilis* NCYC 168, *Candida* sp. 107 NCYC 911 and *C. tropicalis* Zymology Laboratory Collection 3133 (donated by J. Hamilton-Miller). All were maintained on slopes consisting of ($l^{-1}$) 3.0 g malt extract, 3.0 g yeast extract (lab m), 10.0 g glucose, 5.0 g peptone and 20.0 g agar (MYGP; Wickerham, 1951.).

## MEDIA

Unless otherwise stated the yeasts were grown aerobically in medium containing ($l^{-1}$) 20 g glucose, 3.0 g ammonium sulphate, 4.5 g $KH_2PO_4$, 1.0 g yeast extract (lab m), 25 mg $MgSO_4.7H_20$ and 25 mg $CaCl_2.2H_20$, adjusted to pH 4.5. Medium for anaerobic growth, unless stated otherwise, contained ($l^{-1}$) 200 g glucose, 3.0 g ammonium sulphate, 4.5 g $KH_2PO_4$, 4.0 g yeast extract (lab m), 25 mg $MgSO_4.7H_2O$ and 25 mg $CaCl_2.2H_2O$, also adjusted to pH 4.5.

Experiments involving assessment of the effect of ammonium sulphate concentration on production of long-chain alcohols by *C. maltosa* used a defined medium containing a standard vitamin mix to replace yeast extract. The standard vitamin mix consisted of 10 g *m*-inositol, 1.0 g calcium-D-pantothenate, 1.0 g pyridoxin-HCl, 1.0 g thiamin-HCl and 2.0 mg d-biotin, bulked with 500 g

$KH_2PO_4$. The defined medium contained ($l^{-1}$) 4.51 g standard vitamin mix, 20 g glucose, 0.5 g $KH_2PO_4$, 25 mg $MgSO_4.7H_2O$, 25 mg $CaCl_2.2H_2O$ and various concentrations of ammonium sulphate ranging from 0.5 g $l^{-1}$ to 5 g $l^{-1}$. This medium was also adjusted to pH 4.5.

One-litre portions of these media were dispensed into 2-litre round, flat-bottomed flasks and sterilized at 6.89 x$10^4$ Pa for one min. Those flasks used for aerobic growth were fitted with cotton-wool bungs and those used for anaerobic growth were fitted with fermentation locks. A starter culture consisting of 100 ml of the appropriate medium in a 250 ml flask was inoculated with a pinhead of yeast and incubated for 48 h at 30°C on an orbital shaker (200 r.p.m.). A portion of this starter culture containing 10 mg dry wt. yeast was used to inoculate the one-litre portions of medium which were incubated aerobically or anaerobically at 30°C with stirring by a magnetic stirrer (300 r.p.m.).

Cells for mutagenesis experiments were grown in a yeast nitrogen base/defined medium (YNB/DM) consisting of ($l^{-1}$) 3.35 g YNB (Difco), 5.0 g $(NH_4)_2HPO_4$, 0.375 g $Na_2SO_4$ and 1.6 g $KH_2PO_4$, adjusted to pH 6.0 and sterilized at 6.89 x $10^4$ Pa for 15 min. Glucose was sterilized separately, under the same conditions, and added at 20.0 g $l^{-1}$. Various vitamins and inorganic salts were filter-sterilized and added once the YNB had been autoclaved. The vitamin mix consisted of ($l^{-1}$) 0.1 mg d-biotin, 15 mg nicotinic acid, 3 mg calcium-D-pantothenate, 10 mg pyridoxin-HCl and 4 mg thiamin-HCl. The inorganic salts mix consisted of ($l^{-1}$) 5 mg $ZnSO_4$, 5 mg $MnSO_4$ and 5 mg $FeSO_4$. The trace metals mix contained ($l^{-1}$) 1.5 mg $CuSO_4.5H_2O$, 0.2 mg $NaMoO_4$, 0.5 mg $H_3BO_4$, 0.1 mg KI and 0.2 mg $NiSO_4$. A solution of $MgCl_2$ at 2.0 g $l^{-1}$ was also filter-sterilized. The vitamins, inorganic salts, trace-metals and $MgCl_2$ solutions were all added at 10 ml $l^{-1}$ to sterile YNB and 100 ml portions dispensed into 250 ml baffled

flasks. These were inoculated directly with a pinhead of yeast and incubated at 30°C on a rotary shaker (200 r.p.m.).

Growth of all strains of yeast was followed by measuring optical density at 600 nm and relating values to dry wt. yeast with the appropriate standard curve. Viability was estimated using methylene blue. A portion (1.0 ml) of culture diluted to 0.5 mg dry wt. $ml^{-1}$ was stained with methylene blue solution (0.01%, w/v; containing 2%, w/v trisodium citrate) for 10 min; 1000 organisms were scored for uptake of the dye (Fink and Kühles, 1933).

Cells were harvested by centrifugation at 6 000 *g* followed by two washes with water. All centrifugations were carried out at 4°C in a Sorvall RC5B refrigerated superspeed centrifuge (DuPont Company, Willington, Delaware, USA).

## EXTRACTION OF LIPIDS

### Whole Organisms

Lipids were extracted by a modification of the method of Folch *et al.* (1957). After harvesting, the cells were resuspended in 20 ml methanol and shaken in a Braun homogeniser (B. Braun, Melsungen, Germany) for four periods of 30 s with glass beads (40 g; Sigma type V; 0.45 - 0.50 mm diam.) with cooling by expanding $CO_2$. The sample was mixed with chloroform to form a 2:1 (v/v) chloroform: methanol solution and stirred on a flat-bed stirrer (300 r.p.m.) for 2 h. The suspension was filtered through a Whatman no 44 filter paper and the extraction procedure repeated once again on the residue.

The extracts were pooled and shaken vigourously in a separating funnel with a 0.25 vol. of 0.88% (w/v) KCl. The layers were allowed to separate overnight at 4°C and the lower phase removed and taken to dryness with a rotary evaporator. The residue was resuspended in 1 ml chloroform.

**Culture medium**

Organisms were removed from the culture medium by centrifugation (6000*g* ; 5 min; 4°C) and the supernatant filtered through Whatman no 44 filter paper to remove any remaining cells. Lipids were removed from the fluid by shaking vigorously with three successive portions (100 ml) of ethyl acetate. The top layers were removed, the extracts pooled and taken to dryness with a rotary evaporator. The residue was resuspended in 1 ml chloroform.

**LIPID ANALYSIS**

Crude separation of the lipids was achieved using a silicic acid column. The first experiments used the method of White *et al.* (1987), but this was later modified due to subsequent overloading of the column with triacylglycerols contained in extracts from oleaginous yeasts.

The method of White *et al.* (1987), modified from Naccarato *et al.* (1972), used 1 g SIL-LC silicic acid (325 mesh; Lipid Chromatography Grade) packed in a 5 ml glass pipette plugged with glass wool. Even packing was achieved with maximum suction from a vacuum pump accompanied by gentle tapping. The column was saturated with 20 ml hexane, overpressure being applied with high-purity nitrogen. The sample was applied to the top of the column and eluted with

10 ml each of 4%, 7% and 10% diethyl ether in petroleum ether (v/v; b.p. 60 - 80°C). A flow rate of 0.5 ml $min^{-1}$ was maintained and samples collected in 3 ml portions and taken to dryness under a stream of nitrogen gas.

The system was later modified for use with oleaginous yeasts. The column consisted of a 25 ml glass pipette plugged with glass wool as described previously and packed with 5 g SIL-LC silicic acid. Even packing was achieved with a vacuum pump after which the column was saturated with 40 ml hexane (b.p. 67 - 70°C). The sample was applied in 1 ml chloroform and 40 ml hexane used to elute the triacylglycerol fraction. The remaining, more polar, lipids were eluted with 20 ml each of 2%, 10% and 20% diethyl ether in hexane and finally 20 ml 100% diethyl ether (v/v). Fractions were collected in 10 ml portions and taken to dryness under a stream of nitrogen gas.

Appropriate fractions were redissolved in diethyl ether and 20 µl samples streaked on to 20 cm x 20 cm x 0.25 mm Silica Gel 60 precoated TLC plates (Whatman). These were developed in a solvent system consisting of hexane: diethyl ether: acetic acid (80:20:1, by vol). The standards used were tripalmitin, palmitic acid, pentadecanol and ergosterol. The lipids were located by spraying with 0.2% (w/v) 2', 7' - dichlorofluorescein in ethanol and viewed under ultraviolet (254 nm) radiation (Griffith *et al.*, 1981).

Areas containing fatty alcohols were scraped off the plate and the alcohols eluted from the silica gel by three successive extractions with diethyl ether. Long-chain alcohols were separated and identified by GLC after conversion to their trimethylsilyl (TMS) ethers. The sample was taken to dryness under nitrogen gas, then redissolved in 0.3 ml pyridine and an equal volume of bis-(trimethylsilyl) trifluoro-acetimide (BSTFA) added. The sample was then heated in a 1 ml screw-top bottle (Reacti-vial; Pierce) in a dry heating block for 15 min at 70°C.

Samples were identified using a Pye Unicam PU 4500 gas chromatograph fitted with a 25 m QC2/OV101 vitreous silica column. The injection temperature was 300°C and the detector temperature 350°C. The initial column temperature was 190°C and this was raised after 15 min at a rate of 16°C $min^{-1}$ to a final column temperature of 250°C which was maintained for five min. The helium carrier gas had a flow rate of 1 ml $min^{-1}$ and the nitrogen flow rate, as a make up gas across the detector, was 40 ml $min^{-1}$.

Retention times of the samples were compared with the retention times of authentic standards and checked by co-chromatography. The heptadecanol internal standard added at the start of the extraction procedure allowed the fatty alcohol in the sample to be quantified and the peaks were analysed by a Trivector Trio integrator.

## PREPARATION OF CELL-FREE EXTRACTS

*Candida albicans* grown for 72 h in a medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate was used to produce cell-free extracts. The cells (3.5 g dry wt.) were harvested and washed twice in PIPES assay buffer (50 mM; pH 6.5 containing 0.5 mM dithiothreitol and 1.0 mM $MgCl_2$) before suspension in 10 ml assay buffer. The cells were broken with a Braun homogeniser as previously described and suspended in 40 ml assay buffer. The debris was removed by centrifugation at 500 *g* for five min.

The supernatant was centrifuged at 18 000 *g* for 30 min to remove mitochondria or promitochondria. In initial experiments, the microsomal fraction was precipitated with $CaCl_2$ according to the method of Käppeli (1986a). This technique involved adding $CaCl_2$ to 16 mM to the 18 000 *g* supernatant followed

by centrifugation at 15 000 *g* for 15 min. The pellet represented the microsomal fraction and was resuspended in PIPES buffer (50 mM; pH 6.5 containing 150 mM KCl) and recentrifuged at 25 000 *g* for 15 min. The washed pellet was finally resuspended in 2 ml assay buffer.

The supernatant resulting from the microsomal precipitation represented the soluble fraction and the protein was precipitated with ammonium sulphate (55% saturation). The suspension was stirred for 15 min at 4°C and the protein collected by centrifugation at 12 000 *g* for 20 min. The cytosolic protein was redissolved in 2 ml assay buffer.

Later experiments employed centrifugal precipitation of the microsomal fraction in preference to the $CaCl_2$ method. All steps up to the removal of the mitochondria were as already described. However, with this method, the post-mitochondrial supernatant was subjected to centrifugation at 100 000 *g* for 90 min in a MSE Europa 75M ultracentrifuge (MSE Scientific Instruments, Crawley, Sussex, England). The pellet from this centrifugation represented the microsomal fraction and was resuspended in 2 ml assay buffer. The supernatant represented the soluble fraction and the protein was precipitated and resuspended as already described.

## DETERMINATION OF PROTEIN CONTENT

The protein content of fractions was assayed using Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany), a method based on the dye-binding technique of Bradford (1976). Portions (0.1 ml) of the suspension were mixed with 5 ml Bio-Rad dye reagent (20%, v/v, in water). The solution was vortexed and the colour allowed to develop for five min at room temperature. The optical

density was measured at 595 nm and the value related to protein content using a calibration curve constructed with bovine serum albumin.

## ASSAY OF REDUCTION OF PALMITOYL-CoA TO 1-HEXADECANOL

Reduction of palmitoyl-CoA to 1-hexadecanol by cell-free extracts was assayed using a modification of the method described by White *et al.* (1988).The assay consisted of 0.3 µmol NADPH, 0.3 µmol NADH, 1 mg BSA, 2 mg microsomal or soluble protein, 10 nmol [1-$^{14}$C] palmitoyl-CoA (58 mCi mmol$^{-1}$) and 10 nmol unlabelled palmitoyl-CoA in a total volume of 2 ml PIPES assay buffer. The reaction mixture was assembled in a 3 ml Reacti-Vial, flushed with nitrogen gas and incubated for one hour at 30°C with shaking. The reaction was terminated by adding 3 ml methanol and the mixture transferred to a 25 ml separating funnel. The solvent concentration was adjusted to 1:1:2 (by vol.) water: methanol: chloroform and 1-hexadecanol added as a carrier such that 250 nmol existed in each reaction mixture. The mixture was shaken vigorously and the layers allowed to separate overnight at 4°C.

The lower phase was removed and taken to dryness with a rotary evaporator. The sample was redissolved in diethyl ether and applied to a Silica Gel 60 TLC plate (Whatman) in a short streak. The plate was developed twice in a solvent mixture of hexane: diethyl ether: acetic acid (80:20:1, by vol) and the alcohols detected by spraying with 2',7'- dichlorofluorescein and viewed under uv (254 nm) radiation.

The areas containing alcohols were scraped off the plate and eluted as previously described. The samples were dried under nitrogen gas, redissolved in 300 µl BSTFA and heated for 15 min at 70°C. A 250 µl portion of this sample was

added to a vial containing 7.5 ml Optiphase "Safe" scintillant and the radioactivity assayed using a LKB 1217 Rackbeta liquid scintillation counter. A sample (1µl) was analysed by GLC as previously described to assess the extent of carrier recovery. Reductase activity is expressed as nmol palmitoyl-CoA reduced (mg protein)$^{-1}$ h$^{-1}$.

## FRACTIONATION OF CELL HOMOGENATES

Cell breakage to study the subcellular location of fatty alcohols was effected by two methods, namely mechanical disintegration in a Braun homogeniser and osmotic lysis through formation and disruption of spheroplasts.

*Candida albicans* was grown for 168 h in a medium containing (l$^{-1}$) 100 g glucose and 0.5 g ammonium sulphate. The cells were harvested at 6 000 *g* and washed twice in cold water. Cells to be disintegrated by a Braun homogeniser were resuspended in 10 ml Tris-HCl, (50 mM; pH 7.2) containing 0.01M EDTA and 1.0 mM $MgCl_2$ (Buffer A) and shaken with glass beads as already described except that disruption of subcellular organelles was minimized by using only four bursts of 15 s. The homogenate was suspended in a total of 40 ml of the same buffer and whole cells and cell debris removed by centrifugation at 1 000 *g* for five min. The pellet was washed twice with a small volume of buffer. This pellet represented the P1 fraction and the combined supernatants the S1 fraction or cell extract.

Cells to be disintegrated by lysis of spheroplasts were harvested and washed as described for cells disintegrated by Braun homogenisation. The cells were resuspended in 1.4 ml Tris-HCl (pH 8.0) containing 0.01M EDTA per g wet wt. and water added to a total volume of 3.5 ml per g wet wt. of cells. The cells were

pretreated with 0.1 M mercaptoethanol (final concentration) for one hour at 30°C (Lohr, 1988). The sulphydryl agent reduces disulphide bridges in the outer mannan-protein layer and thus aids formation of spheroplasts from stationary-phase cells (Schwencke *et al.*, 1977).

Pretreated cells were harvested at 6 000 *g* for four min and washed twice in water before resuspension at 10 mg dry wt. (ml buffered sorbitol)$^{-1}$ (1.8 M containing 0.05 M Tris, 0.01 M $MgCl_2$ and 0.01 M EDTA; pH 7.2). Zymolyase 10 000 (Kirin Brewery Co., Ltd., Japan) was added at 0.1 mg per 10 mg dry wt. cells and the suspension incubated at 30°C on a rotary shaker (120 r.p.m.). Spheroplast formation was followed by diluting 0.1 ml of the suspension into 2.9 ml water, measuring the optical density at 600 nm and assessing whole cell counts by microscopic examination using a haemocytometer (New Improved Neubaur Haemocytometer; Weber, England).

Complete formation of spheroplasts could not be achieved using stationary-phase cells but, when 70 - 75% digested cells were judged to be osmotically sensitive (85 to 100 min incubation), the cells and spheroplasts were harvested at 1 000 *g* for four min and washed twice in 1.8 M buffered sorbitol. The suspension was diluted to 100 ml with ice-cold distilled water and kept on ice for 20 min. The cells and spheroplasts were subjected to 15 strokes with a teflon glass hand homogeniser (0.1 mm clearance; Henschke *et al.*, 1983) and under these conditions approximately 70% of the spheroplasts broke.

The homogenate was centrifuged at 1 000 *g* for five min and the pellet of whole cells and subcellular debris washed twice in 0.3 M sorbitol. The pellet was termed the P1 fraction and the supernatant the S1 fraction or cell extract.

## Differential Centrifugation of Cell-Free Extracts

The extracts (S1) from cells disrupted by Braun homogenisation or spheroplast lysis were subject to differential centrifugation according to a method adapted from Delaissé *et al.* (1981). The first centrifugation was at 3 500 *g* for five min. The supernatant was decanted and the pellet (P3) washed in either Buffer A (Braun homogenate) or 0.3 M buffered sorbitol (spheroplast lysate). The washing buffer was combined with the S3 supernatant and subjected to centrifugation at 12 000 *g* for 10 min. The washing procedure was repeated and the resulting pellet termed P12 and the supernatant S12. The S12 supernatant was centrifuged at 25 000 *g* for 20 min and the pellet and the combined supernatants termed P25 and S25, respectively. The P100 and S100 fractions were obtained by centrifugation at 100 000 *g* for 90 min. Pellets P3, P12, P25 and P100 were resuspended in 1 ml of the appropriate buffer.

## Extraction of Alcohols following Differential Centrifugation

Fractions obtained from differential centrifugation were heated in 20 ml 80% ethanol at 80°C for 15 min, filtered through Whatman no 44 filter paper and the residue extracted with chloroform: methanol (2:1, v/v) as already described. The lipids were separated using TLC with a solvent system of hexane: diethyl ether: acetic acid (80:20:1, by vol.) and the fatty alcohol content of the fractions determined using GLC. Heptadecanol (250 µg) was added as an internal standard at the start of the extraction.

**Cell Fractionation using Sucrose-Density Gradients.**

Cultures of *C. albicans* (containing 3.5 g dry wt. organisms) grown in medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate for various periods of time were harvested and converted into spheroplasts as already described. When 70 - 75% of the digested cells were judged to be osmotically sensitive, they were harvested at 1 000 *g* for four min and resuspended in a total volume of 30 ml buffered mannitol (0.3 M, pH 7.2). The suspension was then sonicated for 3 x 1-min bursts at 20 Hertz with cooling in an ice bath between bursts.

Discontinuous sucrose gradients consisting of 5 ml each of 60, 55, 50, 45, 40, 35, 30, 25, 20, 15 and 10% sucrose in buffered mannitol (0.3 M, pH 7.2) were constructed and 10 ml of the spheroplast lysate applied to the top of each gradient. The gradients were centrifuged at 50 000 *g* for 120 min at 4°C in an MSE Europa 75M ultracentrifuge (MSE, Scientific Instruments, Crawley, Sussex, England) using a 3 x 70 ml swing-out rotor. Fractions (3 ml) were collected using a peristaltic pump and the optical density at 600 nm of each fraction was measured using an LKB Ultraspec 4050 spectrophotometer. The protein contents of the fractions were assayed as before and the sucrose density in fractions measured using an Atago Hand Refractometer (Type 500; Atago., Ltd., Japan).

Long-chain alcohol contents of the fractions were determined by extracting the fractions overnight with a mixture of chloroform and methanol (2:1, by vol.). Each extract was shaken vigorously in a separating funnel with a 0.25 vol. of 0.88% (w/v) KCl. The layers were allowed to separate at 4°C and the lower phase removed and taken to dryness with a rotary evaporator. The residue was resuspended in a small volume of diethyl ether and applied to a 20 cm x 20 cm x

0.25 mm Silica Gel 60 precoated TLC plate in a short streak. The plate was developed in a solvent system consisting of hexane: diethyl ether: acetic acid (80: 20:1, by vol.) and the long-chain alcohol fraction scraped off, eluted from the silica gel and analysed for long-chain alcohol content as already described.

## MARKER ENZYMES AND COMPONENTS

### Plasma-Membrane ATPase

Activity of vanadate-sensitive ATPase, which is a marker enzyme for the plasma-membrane ATPase in *Sacch. cerevisiae* (Willsky, 1979), was assayed by following the release of Pi from ATP (Cartwright *et al.*, 1987). The reaction mixture consisted of 100 mM MES-Tris buffer (pH 6.5) containing 80 mM KCl, 6 mM $MgCl_2$, 6 mM Na-ATP and enzyme preparation containing 100 µg protein in a final volume of 1 ml. The reaction was started with the addition of Na-ATP. Sodium *ortho*vanadate (100 µM) was included in control mixtures to establish that the enzyme being assayed was inhibited by this compound. The reaction mixture was incubated at 30°C for 30 min and the amount of Pi liberated assessed using the method of Serrano (1978). Addition of 2 ml acidified molybdate solution (2.0%, v/v, conc $H_2SO_4$ containing 0.5%, w/v, ammonium molybdate and 0.5%, w/v, sodium dodecyl sulphate) stopped the reaction. Ascorbic acid (0.02 ml; 10%, w/v) was added and the colour allowed to develop at 30°C for five min. The absorbance of the solution was measured at 750 nm and the Pi concentration determined by comparison to a standard curve. ATPase activity is expressed as µmol Pi liberated (mg protein)$^{-1}$ min$^{-1}$.

**NADPH Cytochrome *c* Oxidoreductase**

This enzyme was assayed according to the method of Honeck *et al.* (1982) by following the increase in absorbance at 550 nm as cytochrome *c* was reduced in the presence of NADPH. The reaction mixture contained, in a final volume of 2 ml, 50 mM Tris-HCl (pH 7.7), 0.1 mM EDTA, 2.2 mM KCN, 0.1 mM NADPH, 0.05 mM cytochrome *c* and 0.1 ml enzyme preparation containing 40 µg protein. The reaction was started by addition of the protein and the absorbance of the reaction mixture measured for three min at room temperature. Enzyme activity was calculated using an absorption co-efficient of 21 µmol $ml^{-1}$ $cm^{-1}$ for cytochrome $c_{red}$ - cytochrome $c_{ox}$. Activity is expressed as nmol cytochrome *c* reduced (mg protein)$^{-1}$ $min^{-1}$.

**Alkaline Phosphatase**

This enzyme was assayed according to Fernandez *et al.* (1981) by following the release of *p*-nitrophenol (NP) from *p*-nitrophenyl phosphate (PNPP). The reaction contained, in a final volume of 2 ml, 100 mM Tris-HCl (pH 8.9), 5 mM $MgCl_2$ and 10 mM PNPP. A portion of enzyme preparation (0.1 ml) containing 100 µg protein was added and the mixture incubated at 30°C for 30 min. The reaction was terminated by adding 1.0 ml NaOH (1.0 M) and the absorbance of the mixture measured at 410 nm. The amount of NP formed was calculated by comparison with a standard curve. Alkaline phosphatase activity is quoted as µmol NP formed (mg protein)$^{-1}$ $min^{-1}$.

**Cytochrome P-450**

This was assayed according to the modified method of Gmünder *et al.* (1981). Cytochrome P-450 content was determined by the reduced CO-difference spectrum using a molar extinction co-efficient of 91 $mmol^{-1}$ $l^{-1}$ $cm^{-1}$. The reaction mixture consisted of 2.5 ml Tris-HCl (50 mM; pH 7.5) containing EDTA (0.1 mM) and 7.5 mg protein. Both the sample and the reference reaction mixtures were reduced with a few grains of sodium dithionite and a baseline established in duplicate. Carbon monoxide was bubbled through the sample cuvette and the spectrum recorded from 400 to 500 nm. Cytochrome P-450 content is expressed as pmol $(mg\ protein)^{-1}$.

**PREPARATION OF PURIFIED CELL WALLS.**

Purified cell walls were prepared by a modification of the methods of McMurrough and Rose (1967) and Fleet (1990). *Candida albicans* was grown for 168 h in a medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate. Cells (1.0g wet wt.) were harvested, washed three times in water and suspended in 20 ml ice-cold Tris-HCl buffer (50 mM, pH 8.5). The suspension was transferred to a Braun bottle containing 30 g glass beads (Sigma type V; 0.45 -0.50 mm diam.) and shaken for six periods each of 30 s in a Braun homogeniser with cooling from expanding $CO_2$. The homogenate was centrifuged at 1 300 *g* for 15 min. The supernatant was retained and the pellet of cell walls and unbroken cells resuspended in 15 ml ice-cold Tris-HCl (pH 8.5). The beads were allowed to settle for one min at 4°C and the supernatant removed. The beads were shaken with a further 15 ml ice-cold buffer, the supernatant removed and combined with the other supernatants, and the mixture centrifuged at 1 300 *g* for 15 min. The pellet was washed five times with cold Tris-HCl, resuspended in ice-

cold water and the suspension centrifuged at 1300 *g* for 20 min. The upper layer of white cell walls was gently resuspended in Tris-HCl buffer leaving the lower layer of yellowish whole cells adhering to the tube. The process was repeated and the wall preparation examined microscopically to ensure that it was free from intact organisms. The pellet was washed 10 times with water and either frozen overnight or used immediately.

Long-chain alcohols were extracted from the cell-wall preparation by heating in 80% (v/v) ethanol at 80°C for 15 min followed by extraction with chloroform: methanol (2:1, v/v). The extracts were analysed for long-chain alcohols as already described.

## MUTAGENESIS

Stationary-phase cultures of *C. tropicalis* were used for all mutagenesis experiments. Cells were grown in 50 ml portions in 250 ml baffled flasks in YNB/DM containing 20 g glucose $l^{-1}$. When in the stationary-phase of growth, cells were harvested, washed and resuspended in 10 ml phosphate buffer (50 mM; pH 7.5). N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) was added to the suspension at a final concentration of 0.5 mg $ml^{-1}$. Portions (2.4 ml) of the culture were removed after 15, 30, 60 and 90 min and added to 10 ml sodium citrate buffer (0.1 M; pH 4.0) to inactivate NTG. The cells were washed twice in phosphate buffer (50 mM; pH 7.5) and resuspended in 10 ml YNB/DM. Glucose was added to a final concentration of 2.5 g $l^{-1}$ and the cells incubated overnight to allow fixation of mutants. The concentration of carbon source allowed at least one cell division but was insufficient to allow significant replication of mutants.

At each time point, total viable counts were estimated by serially diluting 0.1 ml portions of the cell culture in 0.25-strength Ringers solution and plating out 0.1 ml volumes onto potato dextrose agar.

## Quantification of Auxotrophs

The number of auxotrophic mutants generated was estimated by replica plating. Master plates were prepared on potato dextrose agar and these acted as templates for velvet-pad replications onto minimal medium agar. This medium contained ($l^{-1}$) 10.0 g glucose, 5.0 g $(NH_4)_2HPO_4$, 0.375 g $NaSO_4$, 1.6 g $KH_2PO_4$, 20.0 g agar and a 10 ml portion of a trace metals mixture containing ($l^{-1}$) 1.5 mg $CuSO_4.5H_2O$, 0.2 mg $NaMoO_4$, 0.5 mg $H_3BO_4$, 0.1 mg KI and 0.2 mg $NiSO_4$. Any colony failing to grow on this medium was scored as an auxotroph.

## Petite Frequency

The number of petite mutants was estimated from the number of colonies failing to achieve normal colony size when grown on complex medium (potato dextrose agar).

## Selection Procedure

Cells grown aerobically for 120 h in medium containing 0.5 g ammonium sulphate $l^{-1}$ were screened for poor triacylglycerol producers. Cells (50 ml) were harvested, washed twice and resuspended in 5 ml Tris-HCl buffer (0.1 M; pH 7.2). The suspension was applied to a simple gradient consisting of 10 ml 65%

and 20 ml 60% sucrose in Tris-HCl (0.1 M; pH 7.2). The gradients were centrifuged at 3 000 *g* for 30 min at 4°C in a MSE Centaur 1 bench-top centrifuge. Higher density cells banded on the top of the sucrose with a density of 1.25 g $ml^{-1}$ and these were collected and resuspended in medium containing 0.5 g ammonium sulphate $l^{-1}$ and grown aerobically for five days at 30°C. The procedure was repeated twice, allowing the cells to grow for five days between each gradient. After three gradient purifications the band at 1.25g $ml^{-1}$ was more turbid. These cells were plated out on agar containing ($l^{-1}$) 20.0 g glucose, 4.5g $KH_2PO_4$, 1.0 g yeast extract (lab m), 25 mg $CaCl_2.2H_20$, 25 mg $MgSO_4.7H_20$ and 20.0 g agar. Individual colonies were picked off these plates and grown anaerobically for 168 h. Cultures were harvested and the lipids extracted with chloroform: methanol as already described. The crude lipid extracts were taken to dryness under a stream of nitrogen gas, redissolved in 0.3 ml pyridine and 0.1 ml BSTFA added. The extracts were then heated in a 0.8 ml vial in a dry heating block for 5 min at 70°C. Extracts were analysed using a Perkin Elmer 8420 B gas chromatograph fitted with a 8.3 m BP1 wide bore capillary methyl silicone column (SGE) capable of analysing samples containing a proportion of triacylglycerol. The injection system was a programmable temperature vaporiser (PTV) used in the split mode which allows cool injection (50°C) followed by rapid ramping to 380°C in less than 30 sec. The PTV was held at 380°C for four min. The initial column temperature was 100°C and this rose at a rate of 20°C $min^{-1}$ to a final column temperature of 365°C. The detector temperature was 400°C and peaks were analysed using a Perkin Elmer Nelson integrator. Retention times of the samples were compared with the retention times of authentic standards and checked by co-chromatography.

## MATERIALS

All chemicals used were AnalaR grade or of the highest purity available commercially. Reduced nicotinamide adenine dinucleotide phosphate (tetrasodium salt), bovine serum albumin, adenosine 5'-triphosphate (disodium salt), *p*-nitrophenyl phosphate, cytochrome *c*, 2',7'-dichlorofluorescein, bis-(trimethylsilyl) trifluoro-acetamide, pyridine, silicic acid, N-Methyl-N'-nitro-N-nitrosoguanidine and all lipids and lipid standards were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Sodium *ortho*vanadate was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Zymolyase 10 000 was provided by ICN Biomedicals Ltd., High Wycombe, Bucks, U.K. Radioactively labelled palmitoyl-CoA was purchased from Amersham International, Amersham, U.K. Optiphase "Safe" scintillation fluid was obtained from Fisons plc., Scientific Equipment Division, Loughborough, U.K. Gas-liquid chromatography columns were provided by SGE Scientific Glass Engineering Ltd., Milton Keynes, U.K.

# RESULTS

## EFFECTS OF CULTURAL CONDITIONS ON LONG-CHAIN ALCOHOL PRODUCTION BY YEASTS

### Long-chain Alcohol Contents of *Candida albicans* and *Candida maltosa*

Both *C. albicans* and *C maltosa* were found to grow under aerobic and anaerobic conditions, but long-chain alcohol contents of these yeasts were influenced by the culture conditions used (Table 1). Long-chain alcohols were not detected in aerobically-grown *C. maltosa* and contents were low in *C. albicans* under aerobic conditions. Under self-induced anaerobic conditions, alcohol contents were elevated in *C. albicans* and could be detected in *C. maltosa*. The long-chain alcohols detected were tetradecanol, hexadecanol and octadecanol, with trace amounts of dodecanol. No unsaturated long-chain alcohols were detected. The predominant long-chain alcohol in *C. albicans* was hexadecanol although the content of octadecanol was of nearly the same magnitude under these conditions. The predominant alcohol in *C.maltosa* was octadecanol under these conditions. The total long-chain alcohol content of anaerobically grown *C. albicans* was elevated further if the glucose concentration in the medium was lowered from 200 g to 100 g $l^{-1}$. The largest increase occurred in the hexadecanol fraction accompanied by a decrease in the tetradecanol fraction.

**Table 1**

Long-chain alcohol contents of *Candida albicans* and *Candida maltosa* grown under various cultural conditions for 168 h. Values quoted are the means of three separate determinations ± SD. nd indicates alcohols were not detected.

| | **Long-chain alcohol content (ug (g dry wt organisms)$^{-1}$)** | | | |
|---|---|---|---|---|
| | **$C_{14:0}$** | **$C_{16:0}$** | **$C_{18:0}$** | **Total** |
| *Candida albicans* | | | | |
| Aerobic | 30.1 ± 2.3 | 34.8 ± 8.4 | 46.8 ± 1.4 | 111.7 ± 9.2 |
| Anaerobic (100 g glucose $l^{-1}$) | 117.5 ± 13.5 | 595.3 ± 50.4 | 280.8 ± 26.4 | 993.6 ± 48.7 |
| Anaerobic (200 g glucose $l^{-1}$) | 161.5 ± 8.5 | 242.7 ± 14.5 | 228.3 ± 9.6 | 632.5 ± 48.7 |
| *Candida maltosa* | | | | |
| Aerobic | nd | nd | nd | nd |
| Anaerobic (200 g glucose $l^{-1}$) | 39.5 ± 6.3 | 53.2 ± 3.2 | 96.1 ± 9.9 | 188.8 ± 9.9 |

## Long-chain Alcohol Contents of *Pichia fermentans*

Long-chain alcohols could not be detected in *P. fermentans* after aerobic growth (Table 2). Tetradecanol and hexadecanol were detected in this yeast after self-induced anaerobic growth but amounts were small. Octadecanol was not detected in this yeast under these conditions.

## Growth and Production of Long-Chain Alcohols by *Candida* sp. 107 under Self-induced Anaerobic Conditions

*Candida* sp. 107 had a mean generation time of 2 h 10 min and organisms reached the stationary-phase of growth after 48 h with a final growth yield of 2.0 mg dry wt. $ml^{-1}$ (Fig. 1). Maximum contents of long-chain alcohol occurred in the stationary-phase of growth with maximum values at 120 h. After this time the contents of all alcohols declined. Tetradecanol and hexadecanol were found to be the major alcohols and octadecanol was found at smaller concentrations throughout the growth of the culture. The maximum rates of increase were found to be 2.6 $\mu g\ h^{-1}$ for tetradecanol, 1.8 $\mu g\ h^{-1}$ for hexadecanol and 0.9 $\mu g\ h^{-1}$ for octadecanol (Fig. 1).

## Effect of Ammonium Sulphate Concentration on Long-Chain Alcohol Production by *Candida maltosa*

The concentration of the nitrogen source in the medium has been shown to have an effect on lipid production in a number of yeasts (Rattray *et al.*, 1975). Hence a study was made into the effect of varying ammonium sulphate concentration in the medium of aerobically-grown *C. maltosa* . At high ammonium sulphate

**Table 2**

Long-chain alcohol contents of *Pichia fermentans* grown under aerobic and anaerobic conditions for 168 h. Values quoted are the means of three separate determinations ± SD. nd indicates alcohols were not detected.

| Growth Condition | Long-chain Alcohol Content (ug (g dry wt organisms)$^{-1}$) | | | |
|---|---|---|---|---|
| | $C_{14:0}$ | $C_{16:0}$ | $C_{18:0}$ | Total |
| Anaerobic | 26.7 ± 0.5 | 34.5 ± 2.3 | nd | 61.2 ± 3.3 |
| Aerobic | nd | nd | nd | nd |

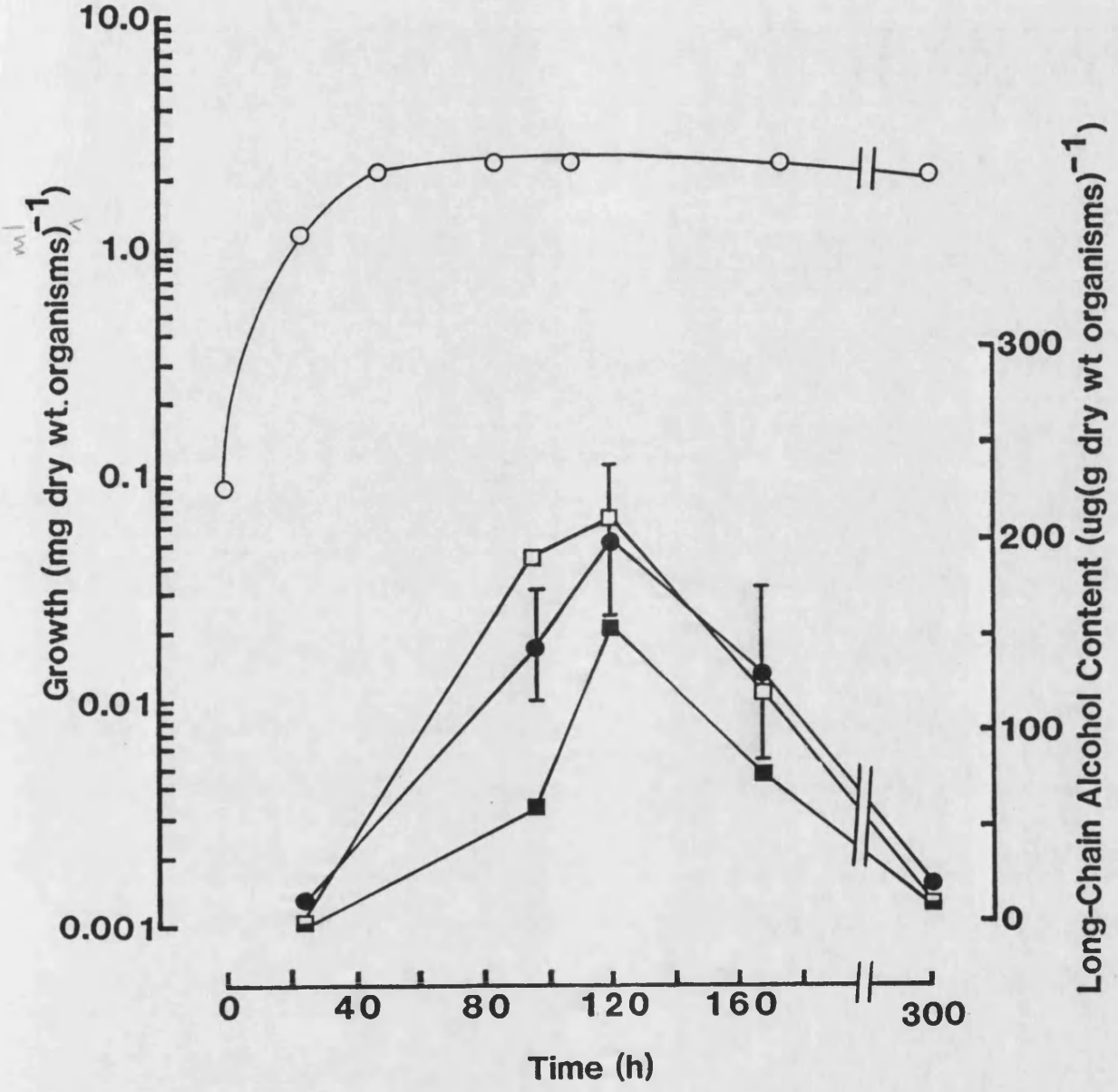



**FIGURE 1.** Time-course of self-induced anaerobic growth (○) of *Candida* sp. 107 and of its contents of $C_{14:0}$ (●), $C_{16:0}$ (□) and $C_{18:0}$ (■) long-chain alcohols. Values plotted are the means of three independent determinations. SD values are given for contents of $C_{16:0}$ alcohol only.

concentrations long-chain alcohols were not detected (data not shown). However, as the concentration of the nitrogenous nutrient was lowered long-chain alcohols could be detected (Fig. 2). This content was elevated if the glucose concentration in the medium was raised from 20 g $l^{-1}$ to 200 g $l^{-1}$. At concentrations of ammonium sulphate of 3.0 g $l^{-1}$ the major alcohol was tetradecanol, but at lower concentrations the major alcohol was hexadecanol. When the glucose concentration was raised to 200 g $l^{-1}$ in conjunction with a low ammonium sulphate concentration, the major long-chain alcohol was octadecanol. However, contents throughout were of a low value.

**Effect of Ammonium Sulphate Concentration on Long-Chain Alcohol Production by *Candida albicans***

White *et al.* (1988) established that, using the medium employed in this work for self-induced anaerobic growth, the concentration of glucose which induced maximum long-chain alcohol production by *C. albicans* was 100 g $l^{-1}$. A study of the effect of ammonium sulphate concentration on long-chain alcohol production showed that a concentration of 0.5 g $l^{-1}$ gave considerably greater total long-chain alcohol production (Fig. 3). Lower concentrations were not employed in this study since they decreased the production of yeast mass. Lowering the ammonium sulphate concentration also had an influence on the relative amounts of the three fatty alcohols detected. At a concentration of ammonium sulphate of 3.0 g $l^{-1}$, the predominant alcohol was hexadecanol. However, at lower concentrations the major alcohol was tetradecanol and contents of octadecanol were decreased.

**FIGURE 2.** Effect of ammonium sulphate concentration on long-chain alcohol contents of *Candida maltosa* grown under aerobic conditions for 168 h. The number at the base of each bar indicates saturated alcohol chain-length. Each bar represents the mean of three independent determinations ± SD.

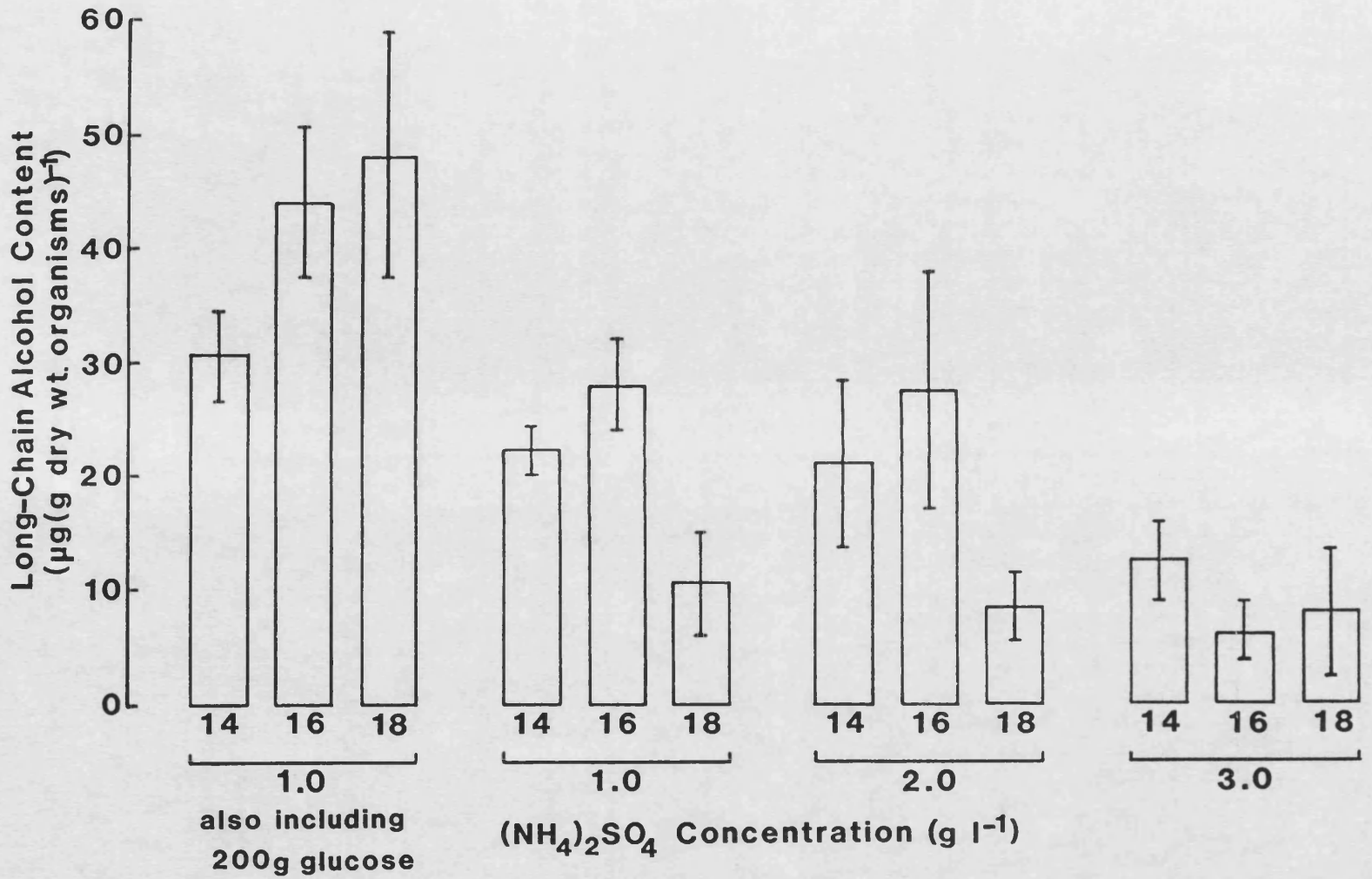
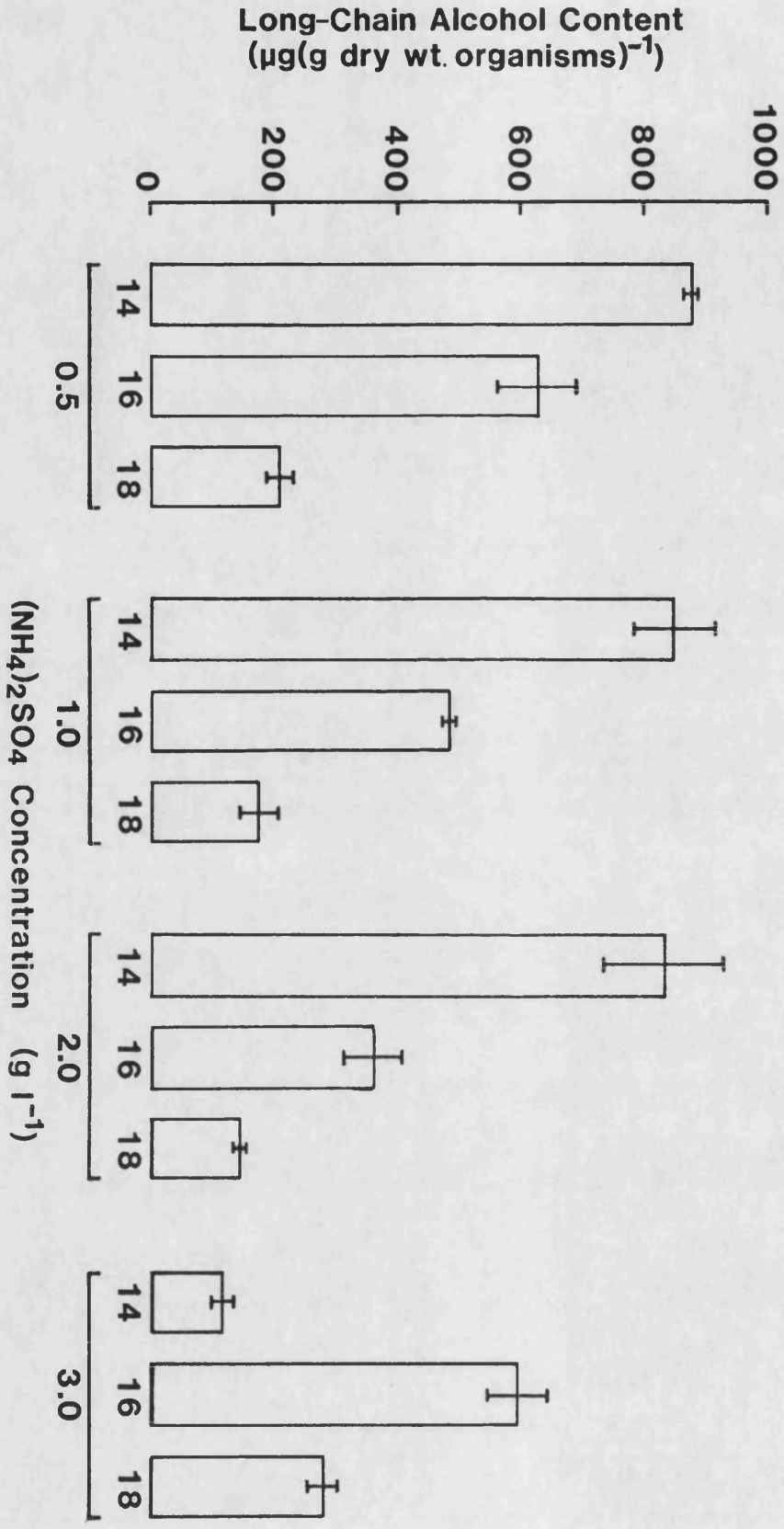

Long-Chain Alcohol Content
(µg(g dry wt. organisms)$^{-1}$)
60
50
40
30
20
10
0
14
16
18
1.0
also including
200g glucose
1.0
2.0
3.0
$(NH_4)_2SO_4$ Concentration (g $l^{-1}$)

**FIGURE 3.** Effect of ammonium sulphate concentration on long-chain alcohol contents of *Candida albicans* grown under self-induced anaerobic conditions for 168 h. The number at the base of each bar indicates saturated alcohol chain-length. Each bar represents the mean of three independent determinations ± SD.


Long-Chain Alcohol Content
(µg(g dry wt. organisms)$^{-1}$)
0
200
400
600
800
1000
0.5
1.0
2.0
3.0
14
16
18
$(NH_4)_2SO_4$ Concentration (g $l^{-1}$)

## Effect of Ammonium Sulphate Concentration on Long-chain Alcohol Contents of *Candida utilis* and *Candida tropicalis*

The preceeding study showed that maximum long-chain alcohol contents of *C. albicans* were obtained with a glucose concentration of 100 g $l^{-1}$ and an ammonium sulphate concentration of 0.5 g $l^{-1}$; thus *C. utilis* and *C. tropicalis* were both grown under these cultural conditions (Table 3). Long-chain alcohol contents of *C. tropicalis* were measurable, but low. The total long-chain alcohol content of *C.utilis* was considerably higher, but was three-fold less than that obtained for *C. albicans* under the same cultural conditions.

## Transfer of Stationary-Phase Cells into Fresh Medium.

Anaerobically grown cultures of *C. albicans* grown for 168 h in medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate (high long-chain alcohol content) or 20 g glucose and 3 g ammonium sulphate (low long-chain alcohol content) were used for these studies.

When organisms with a high long-chain content were harvested and portions (0.8 g dry wt.) resuspended in 1 l of fresh medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate, growth occurred only after a lag phase of approximately 12 h (Fig. 4). During this time, the content of long-chain alcohols fell from a value of about 1700 µg (g dry wt. organisms)$^{-1}$ to around 500 µg (g dry wt. organisms)$^{-1}$ (Fig. 5). Minimum contents of long-chain alcohols occurred at approximately 16 h. Organisms entered the stationary-phase of growth after approximately 24 h (Fig. 4) and this was accompanied by synthesis of additional tetradecanol, hexadecanol and, to a lesser extent,octadecanol. When organisms

**Table 3**

Long-chain alcohol contents of yeasts grown under anaerobic conditions in medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate. Values quoted are the means of three separate determinations ± SD

| **Species** | **Long-chain Alcohol Content (ug (g dry wt organisms)$^{-1}$)** | | | |
|---|---|---|---|---|
| | **$C_{14:0}$** | **$C_{16:0}$** | **$C_{18:0}$** | **Total** |
| *Candida utilis* | 196.1 ± 13.2 | 232.5 ± 23.9 | 155.1 ± 19.0 | 583.7 ± 22.1 |
| *Candida tropicalis* | 14.5 ± 0.7 | 17.5 ± 1.3 | 5.3 ± 0.5 | 37.3 ± 0.9 |

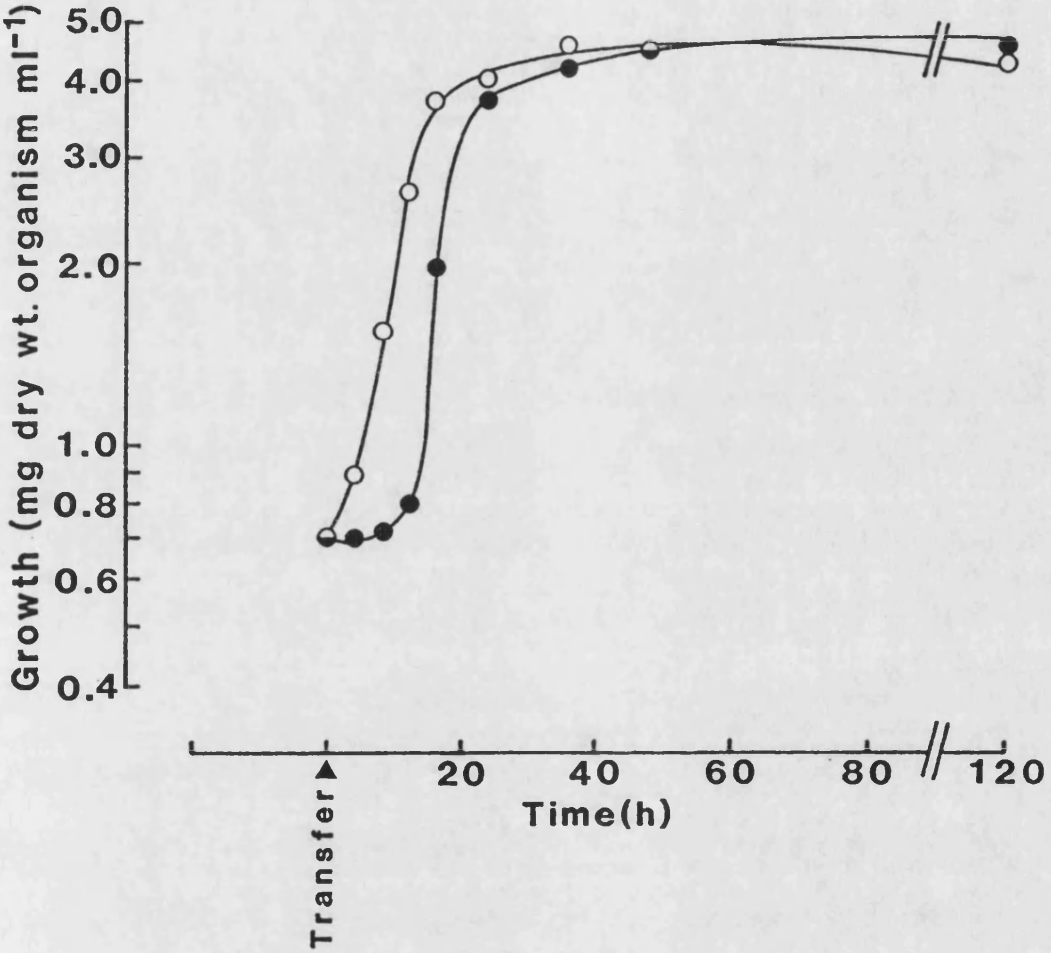



**FIGURE 4.** Time-course of the growth of *Candida albicans* grown under self-induced anaerobic conditions for 168 h in medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate (●) or medium containing 20 g glucose and 3.0 g ammonium sulphate(○) and then transferred to fresh medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate.

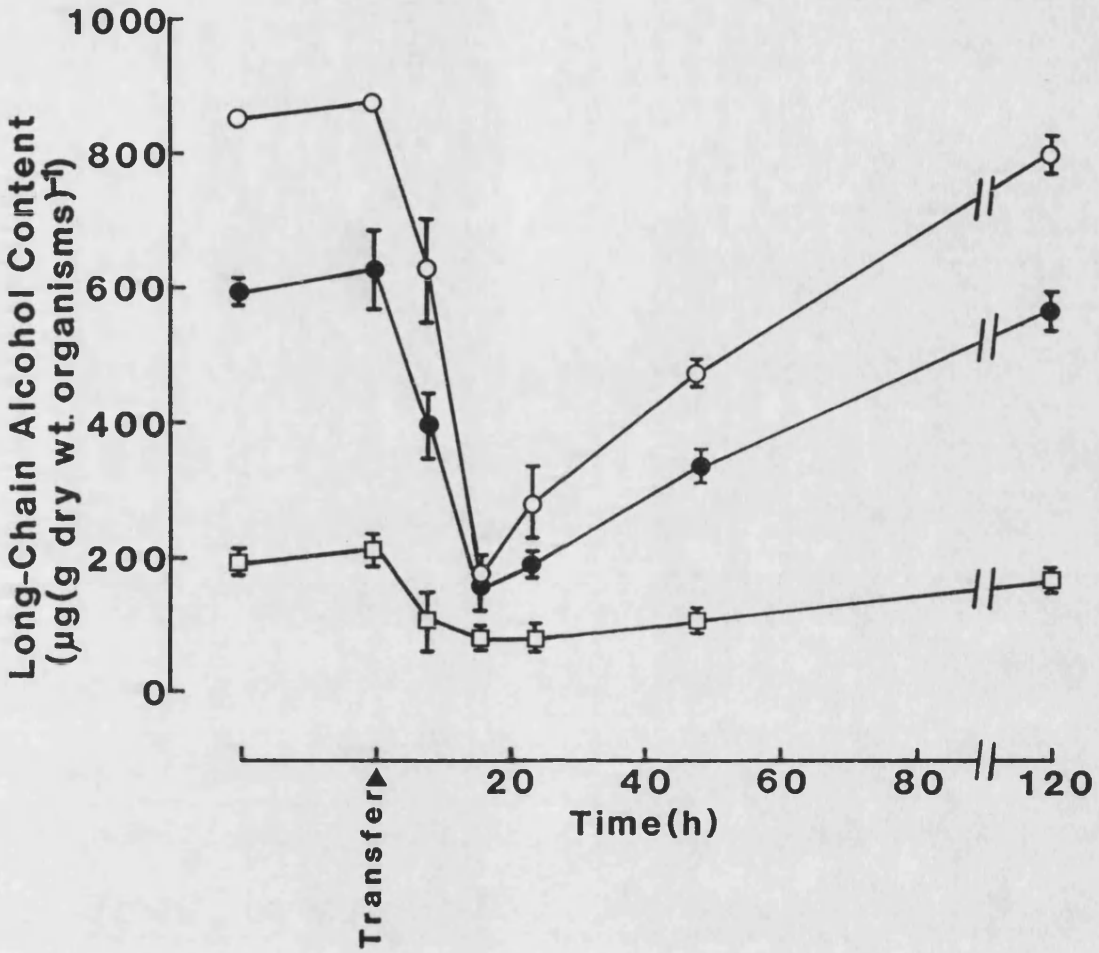



**FIGURE 5.** Changes in contents of $C_{14:0}$ (○), $C_{16:0}$ (●) and $C_{18:0}$ (□) long-chain alcohols in *Candida albicans* grown under self-induced anaerobic conditions for 168 h in medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate and then transferred to fresh medium of the same composition. Values quoted are the means of three separate determinations ± SD.

with a low long-chain alcohol content were resuspended in fresh medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate, the lag phase of growth was very much shorter and organisms entered the stationary-phase of growth after approximately 16 h.

For double-transfer experiments, the cells were allowed to grow at 30°C for 48 h after the initial transfer. The culture was harvested at this time and portions (0.8 g dry wt.) resuspended in 1 l of fresh medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate (Fig. 6). The long-chain alcohol content fell once more to a value of approximately 500 μg (g dry wt organisms)$^{-1}$, 16 h after transfer. This was followed by resynthesis of long-chain alcohols at a slower rate than after the first transfer.

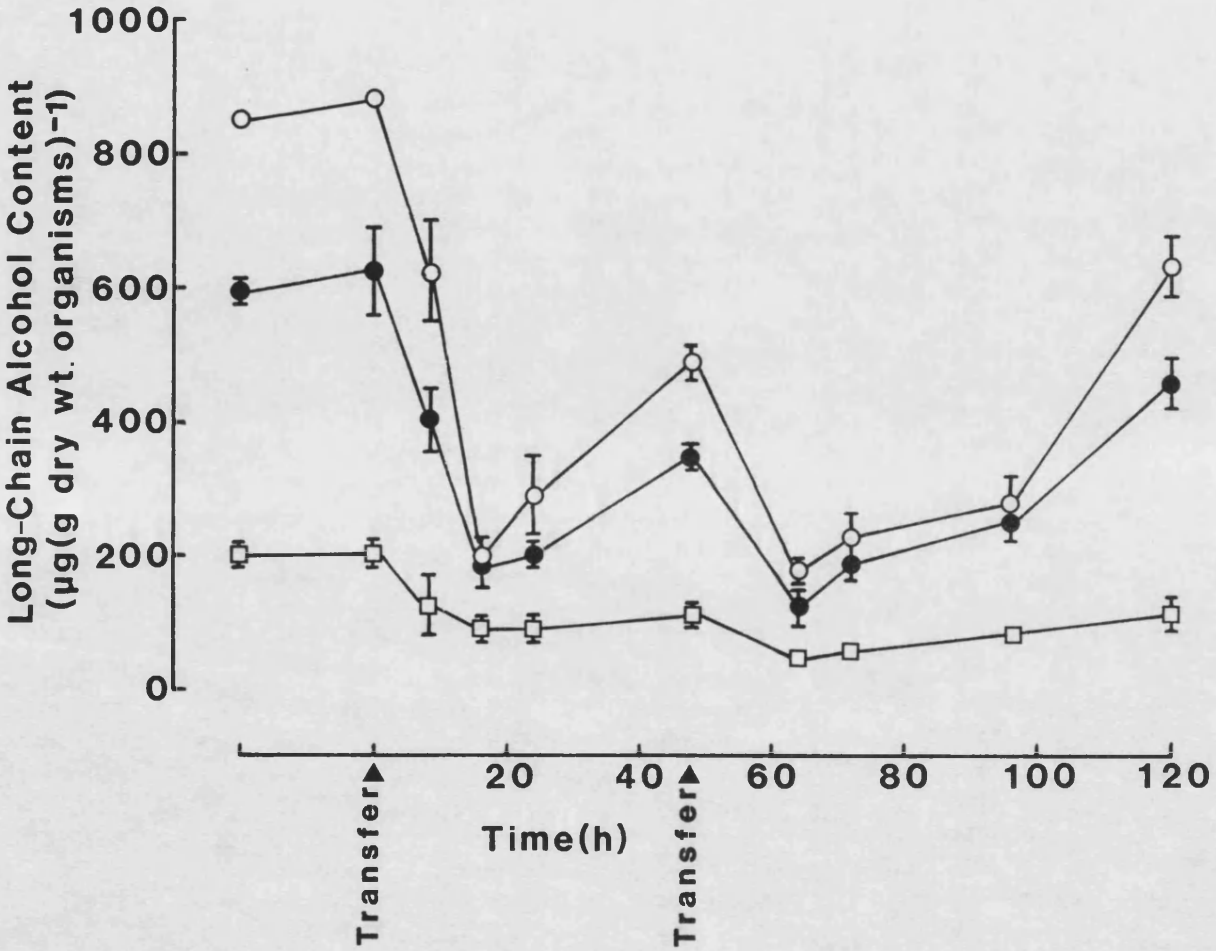
Long-chain alcohols could not be detected in the medium of 168-h cultures when first transferred to fresh medium, but as the long-chain alcohol content of the organisms fell, long-chain alcohols were detected in the medium (Fig. 7). Tetradecanol was detected in the highest concentration with lower concentrations of hexadecanol and octadecanol. The long-chain alcohol content of the medium rose sharply for 24 h following transfer and at a slower rate thereafter, although contents were higher after 120 h.

## REDUCTION OF [1-$^{14}$C] PALMITOYL-CoA BY CELL-FREE EXTRACTS OF *Candida albicans*

### Development of Assay

The initial experiments to study the conversion of palmitoyl-CoA to 1-hexadecanol by cell-free extracts of *C. albicans* involved $CaCl_2$ precipitation of

**FIGURE 6.** Changes in the contents of $C_{14:0}$ (○), $C_{16:0}$ (●) and $C_{18:0}$ (□) long-chain alcohols of *Candida albicans* grown under self-induced anaerobic conditions for 168 h in medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate and then transferred to fresh medium of the same composition. After 48 hours the cells were transferred for a second time to fresh medium of the same composition. Values quoted are the means of three independent determinations ± SD.


Long-Chain Alcohol Content
(μg(g dry wt. organisms)-1)
1000
800
600
400
200
0
20
40
60
80
100
120
Time(h)
Transfer
Transfer

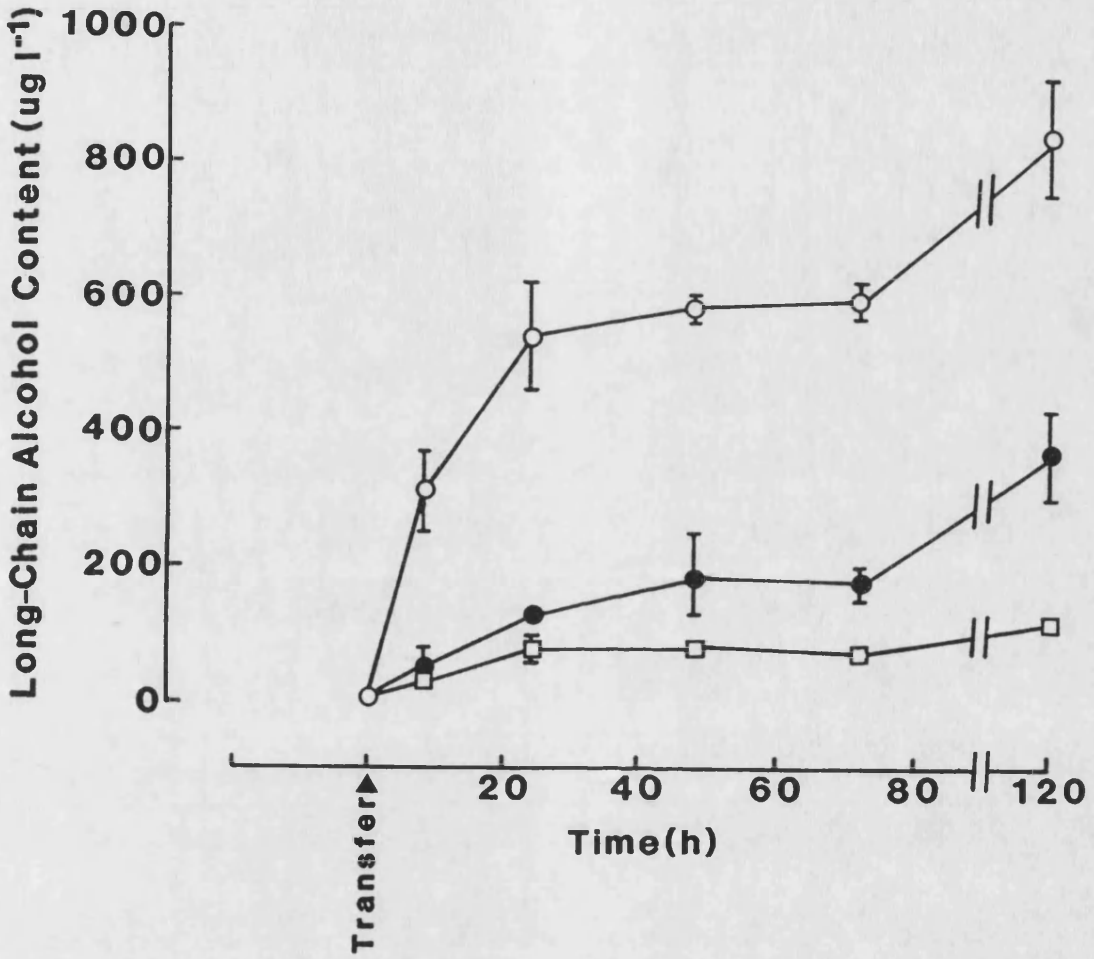



**FIGURE 7.** Changes in the contents of $C_{14:0}$ (○), $C_{16:0}$ (●) and $C_{18:0}$ (□) long-chain alcohols in the medium of *Candida albicans* grown under self-induced anaerobic conditions for 168 h in medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate and then transferred to fresh medium of the same composition. Values quoted are the means of three separate determinations ± SD.

the microsomal fraction. Table 4 shows the cofactor requirements for this reduction. Maximum rates of conversion were obtained with the microsomal fraction in the presence of both NADH and NADPH. The absence of NADH from the assay mixture caused only a very slight decrease in the rate of [1-$^{14}$C] hexadecanol formation, but the absence of NADPH caused a substantial decrease in activity. The cofactor requirement of the soluble fraction shows a similar pattern although the activity in this fraction is lower than in the microsomal fraction. In both cases there was no conversion of palmitoyl-CoA to hexadecanol when the protein preparation was boiled before addition to the enzyme assay.

These initial experiments involved the precipitation of the microsomal fraction by $CaCl_2$ by a modification of a method of Käppeli (1986a). A comparison was made between this method of obtaining a microsomal fraction and a method involving centrifugation of the post-mitochondrial cell-free extract at 100 000 *g* (Table 5). Following centrifugal precipitation a higher value was obtained for reductase activity in the microsomal fraction (Table 5) than that obtained following $CaCl_2$ precipitation (Table 4). The addition of $CaCl_2$ to the 100 000*g* precipitate caused a drop in activity indicating a possible inhibitory effect of this compound. The reductase activity in the soluble fraction was also slightly elevated following centrifugal precipitation of the microsomes.

An increase in palmitoyl-CoA concentration in the assay caused an increase in the rate of hexadecanol formation by the microsomal fraction (Table 5). This was an indication that an assay containing 8 nmol of palmitoyl-CoA was substrate limited, although only 0.045 nmol hexadecanol (mg protein)$^{-1}$ h$^{-1}$ was produced. Subsequent assays contained 20 nmol palmitoyl-CoA in a final assay volume of 2 ml. Approximately 30% of the added radioactivity was recovered as the free fatty acid (results not shown), but free fatty aldehydes were not detected.

**Table 4**

Enzymic reduction of [1-$^{14}$C] palmitoyl-Co A by fractions obtained by calcium chloride precipitation of microsomes from a post-mitochondrial cell-free extract of *Candida albicans*. The complete system is described under Methods and contained 8 nmol substrate. Values quoted are the means of three separate determinations ± SD.

| **Incubation System** | **Hexadecanol Produced (nmoles $h^{-1}$(mg protein)$^{-1}$)** | |
|---|---|---|
| | Soluble Fraction | Microsomal Fraction |
| Complete system | 0.022 ± 0.003 | 0.038 ± 0.004 |
| Complete system minus NADH | 0.020 ± 0.003 | 0.036 ± 0.006 |
| Complete system minus NADPH | 0.009 ± 0.001 | 0.011 ± 0.001 |
| Complete system + boiled enzyme | 0.000 | 0.001 ± 0.000 |

**Table 5**

Enzymic reduction of [1-$^{14}$C] palmitoyl-CoA by fractions obtained by centrifugation at 100 000 *g* of a mitochondria-free cell-free extract of *Candida albicans*. The complete system is described under Methods and contained 8 nmol substrate, unless stated otherwise. Values quoted are from a single experiment.

| **Incubation System** | **Hexadecanol Produced (nmoles $h^{-1}$(mg protein)$^{-1}$)** |
|---|---|
| Microsomal fraction | 0.045 |
| Microsomal fraction +16 nmol substrate | 0.096 |
| Microsomal fraction + 16 mM $CaCl_2$ | 0.028 |
| Soluble fraction | 0.029 |

As the amount of microsomal protein was increased in the assay mixture the production of labelled fatty alcohol increased up to 2.0 mg total protein $ml^{-1}$ (Fig. 8). Any further increase in protein concentration did not lead to a corresponding increase in reductase activity. The standard assay contained 1.0 mg total protein $ml^{-1}$ and as such avoided substrate limitation.

This was confirmed by a series of assays containing a range of substrate concentrations (Fig. 9). An increase in palmitoyl-CoA concentration up to 8 nmol $ml^{-1}$ caused an increase in palmitoyl-CoA reductase activity. Above 8 nmol $ml^{-1}$ no further increase in activity occurred. The standard assay (2 ml total vol.) contained 10 nmol palmitoyl-CoA $ml^{-1}$.

**Properties of the Enzyme**

The curve obtained for activity over a range of substrate concentrations was of a sigmoid shape (Fig. 9) allowing only an approximation of the $K_m$ value. The apparent $K_m$ obtained for the reductase activity was 20 nmoles (Fig. 9 insert).

The effect of pH on reductase activity was tested with a microsomal fraction of a cell-free extract of *C. albicans*. A bell-shaped curve showed maximal activity at pH 6.6. Activities at pH 6.0 and 7.5 were approximately 30% of the maximum (Fig. 10).

The rate of hexadecanol formation was linear for about 45 min without any appreciable alteration in the initial velocity. After this time the rate slowed and no further formation of hexadecanol could be detected after 75 min (Fig. 11).

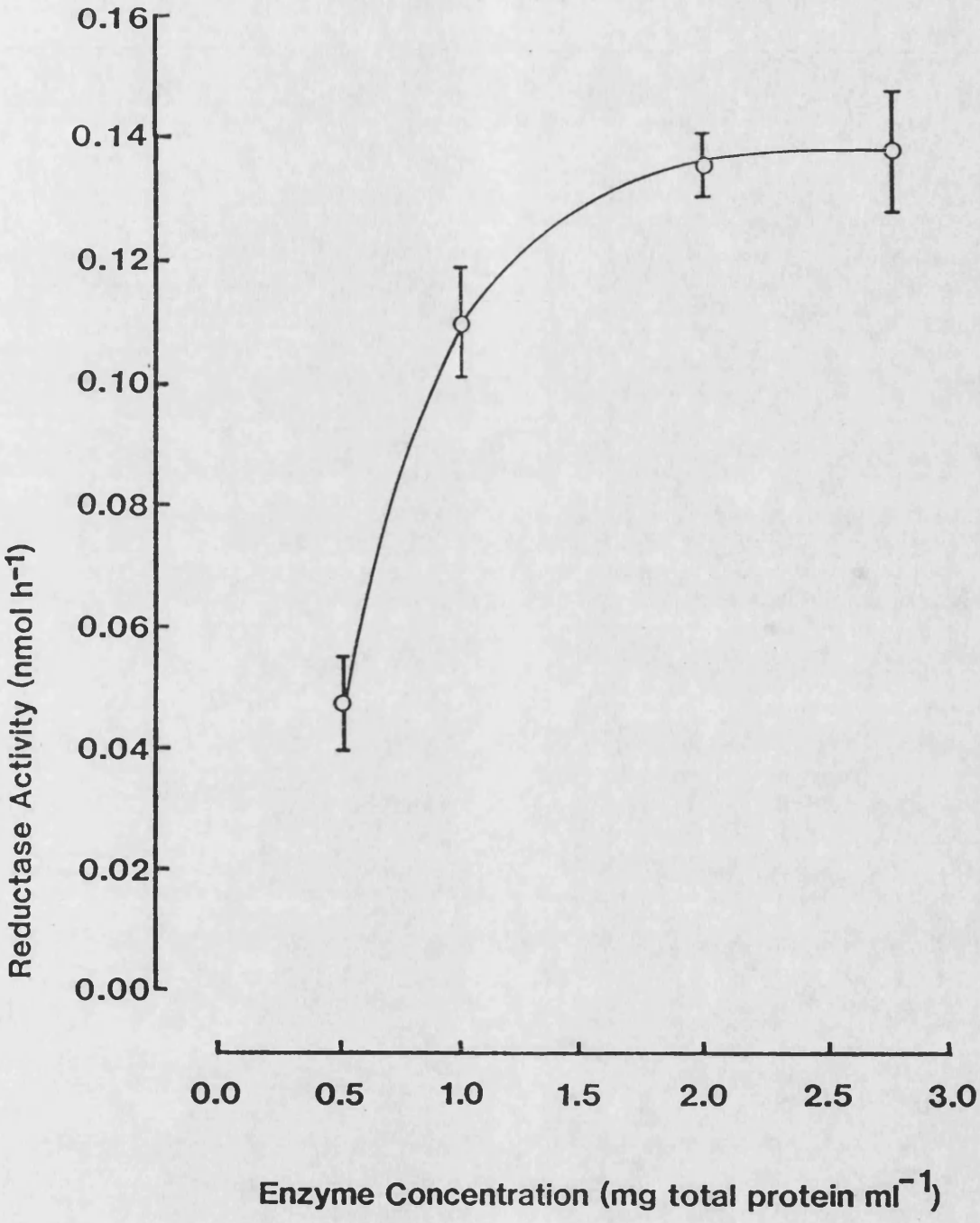



**FIGURE 8.** The effect of enzyme concentration on the activity of palmitoyl-CoA reductase in a cell-free extract of *Candida albicans* grown for 72 h. Values quoted are the means of three independent determinations ± SD.

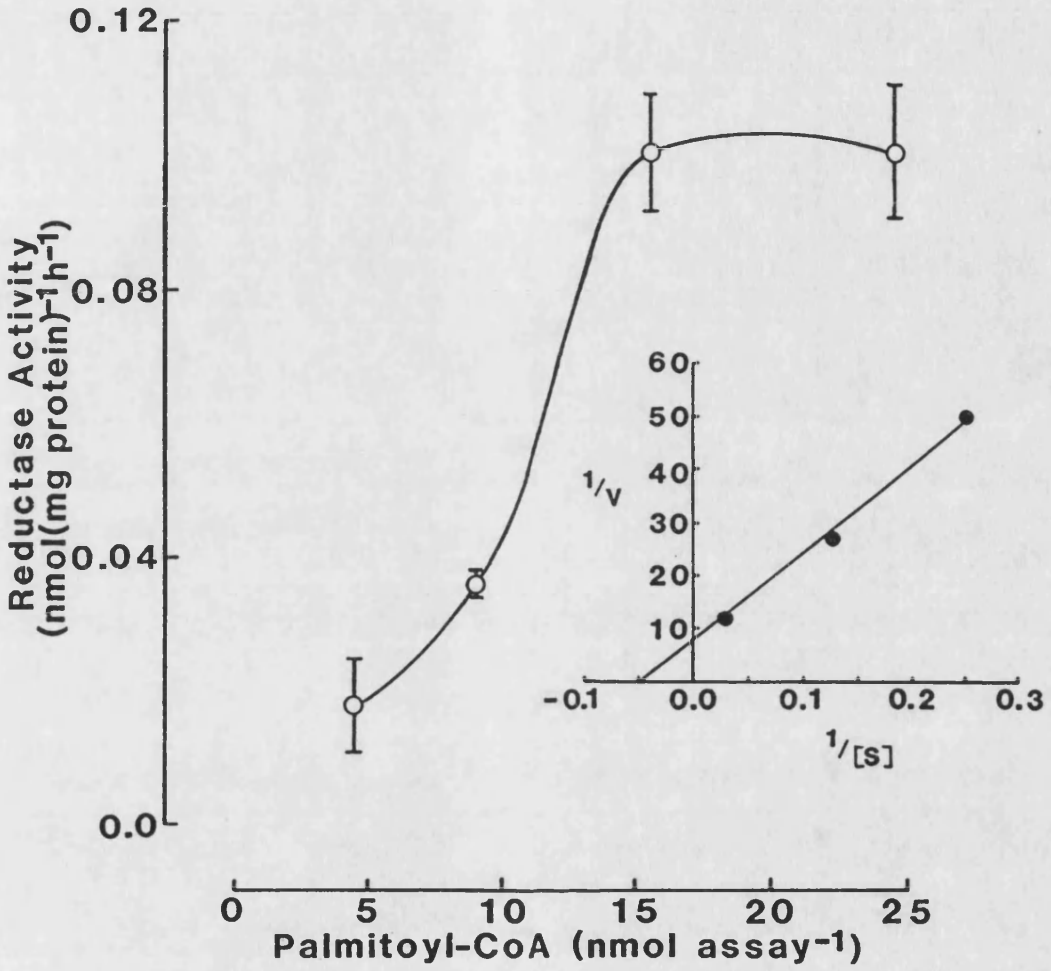



**FIGURE 9.** Palmitoyl-CoA dependence of palmitoyl-CoA reductase from a cell-free extract of *Candida albicans* grown for 72 h. Values quoted are the means of three independent determinations ± SD. Insert: Linewear-Burk plot of palmitoyl-CoA reductase activity. Values quoted are the means of three independent determinations.

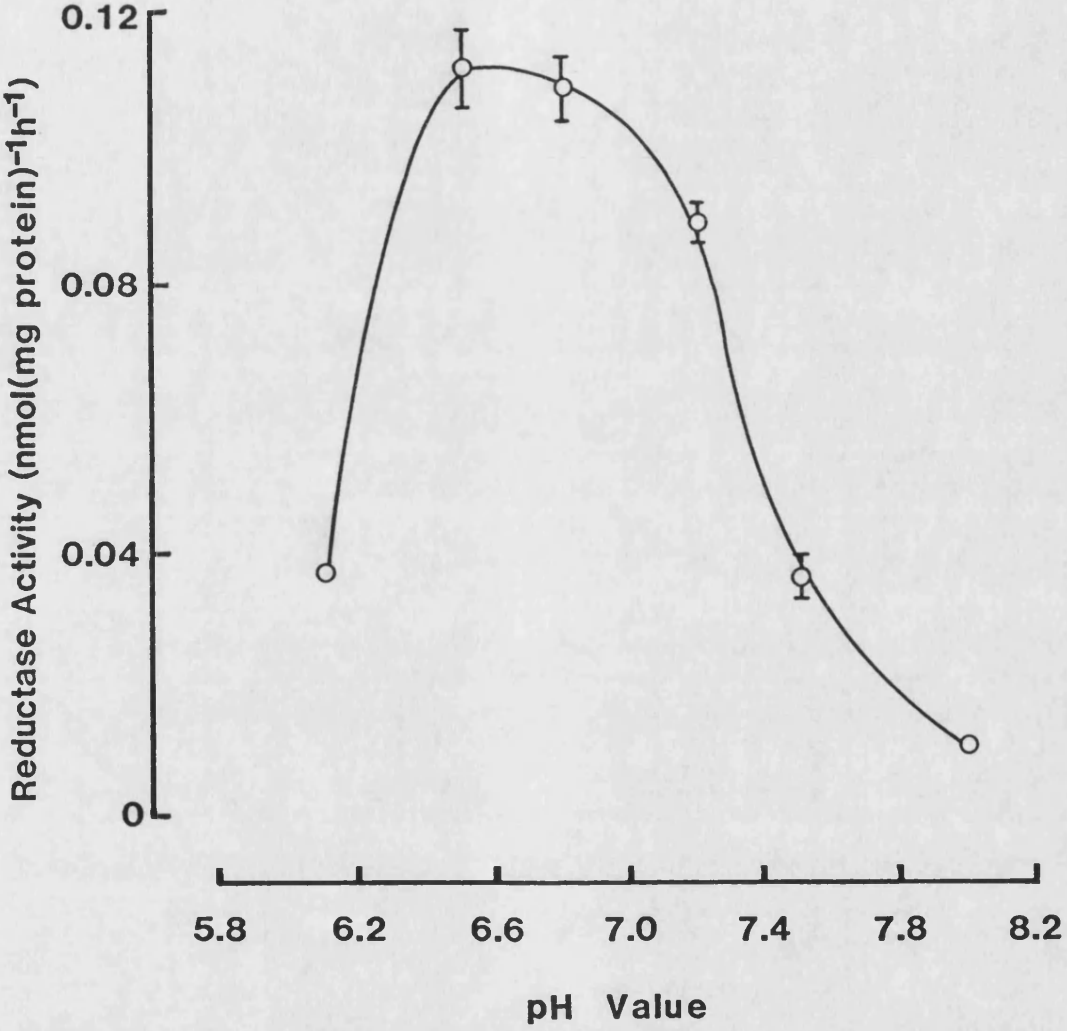



**FIGURE 10.** Effect of pH value on the activity of palmitoyl-CoA reductase from a cell-free extract of *Candida albicans* grown for 72 h. Values quoted are the means of three independent determinations ± SD.

### Changes in Reductase activity during growth of *Candida albicans*

A study was made of palmitoyl-CoA reductase activity over the time-course of hexadecanol production (Fig. 12). Growth of *C. albicans* over the time-course is also shown. Palmitoyl-CoA activity and hexadecanol content were not detectable during exponential growth of the organism, but there was a very rapid increase in palmitoyl-CoA reductase activity once the organism had entered the stationary-phase of growth. The peak of reductase activity coincided with the maximum rate of production of hexadecanol at 72 h. However, the enzyme activity began to decline after 96 h when the hexadecanol content of the organisms was still increasing.

## SUBCELLULAR LOCATION OF LONG-CHAIN ALCOHOLS

### Formation and Stability of Spheroplasts from Self-induced Anaerobically-grown *Candida albicans*

The age of cells used for production of spheroplasts from *C. albicans* had an effect on the extent of spheroplast formation (Fig. 13). Young cells were very susceptible to treatment with the cell wall-degrading enzyme preparation (Zymolyase) and more than 90% of cells harvested in the exponential phase of growth could be converted into spheroplasts. Once cells entered the stationary-phase of growth, they became more resistant to attack by Zymolyase and only a lower rate of conversion into spheroplasts could be achieved. This resistance increased slightly in late stationary-phase cells.

The concentration of buffered sorbitol used as an osmotic stabilizer during formation of spheroplasts had a considerable effect on their stability (Fig. 14).

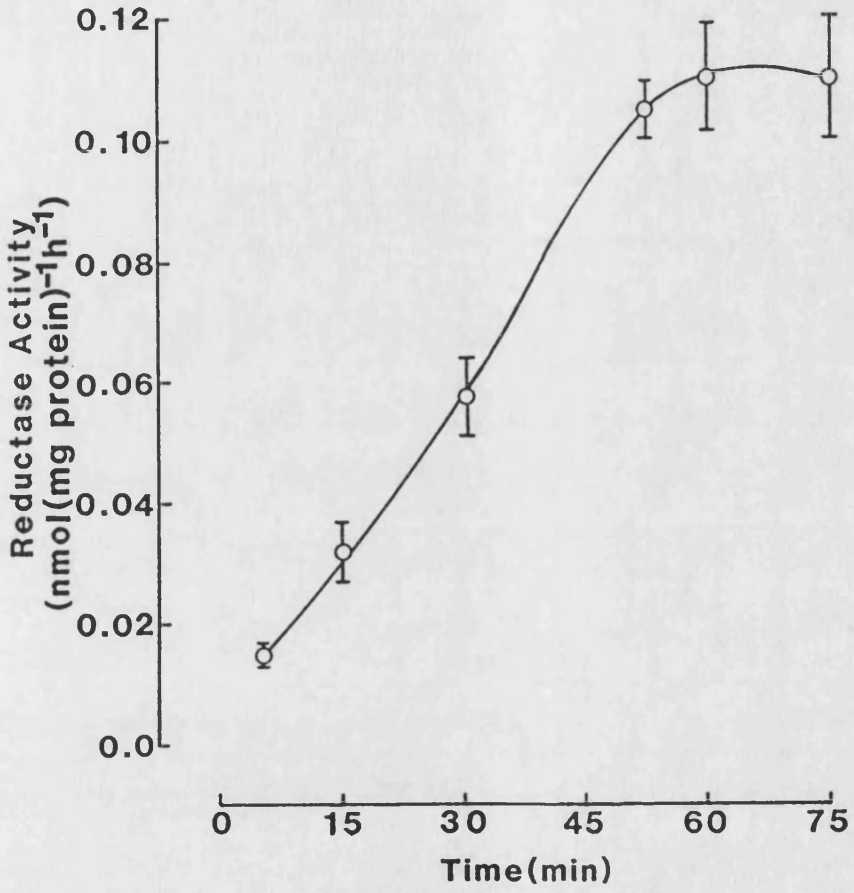
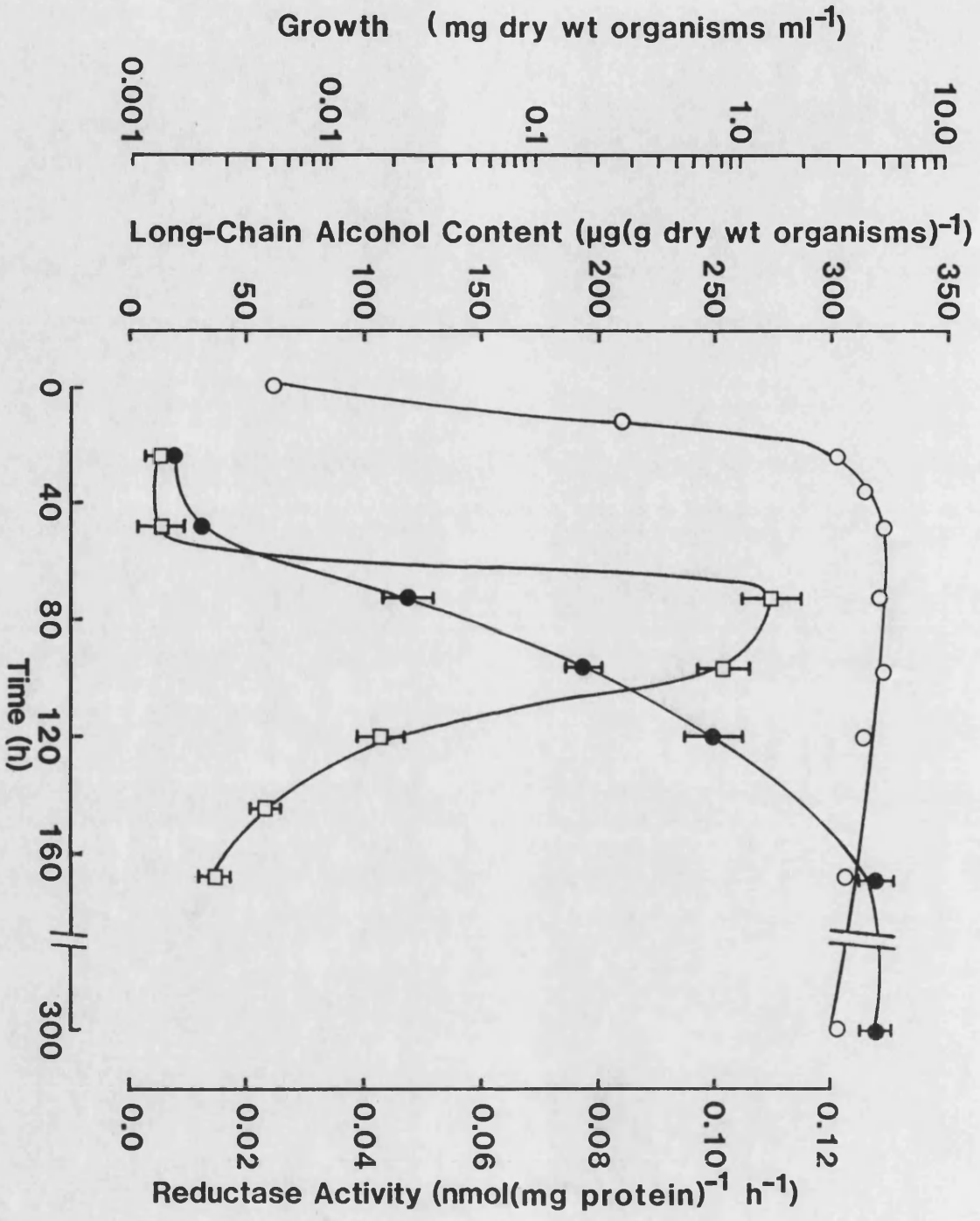



**FIGURE 11.** Time-course of formation of $C_{16:0}$ long-chain alcohol from [1-$^{14}$C] palmitoyl-CoA by a cell-free extract of *Candida albicans* grown under self-induced anaerobic conditions for 72 h. Values quoted are the means of three independent determinations ± SD.

**FIGURE 12.** Time-course of self-induced anaerobic growth (○) and $C_{16:0}$ long-chain alcohol content (●) of *Candida albicans* and palmitoyl-CoA reductase activity (□) of a cell-free extract of this yeast. Values quoted for long-chain alcohol content and reductase activity are the means of three independent determinations ± SD.


Growth (mg dry wt organisms ml$^{-1}$)
0.001
0.01
0.1
1.0
10.0
Long-Chain Alcohol Content (µg(g dry wt organisms)$^{-1}$)
0
50
100
150
200
250
300
350
Time (h)
0
40
80
120
160
300
0.0
0.02
0.04
0.06
0.08
0.10
0.12
Reductase Activity (nmol(mg protein)$^{-1}$ h$^{-1}$)

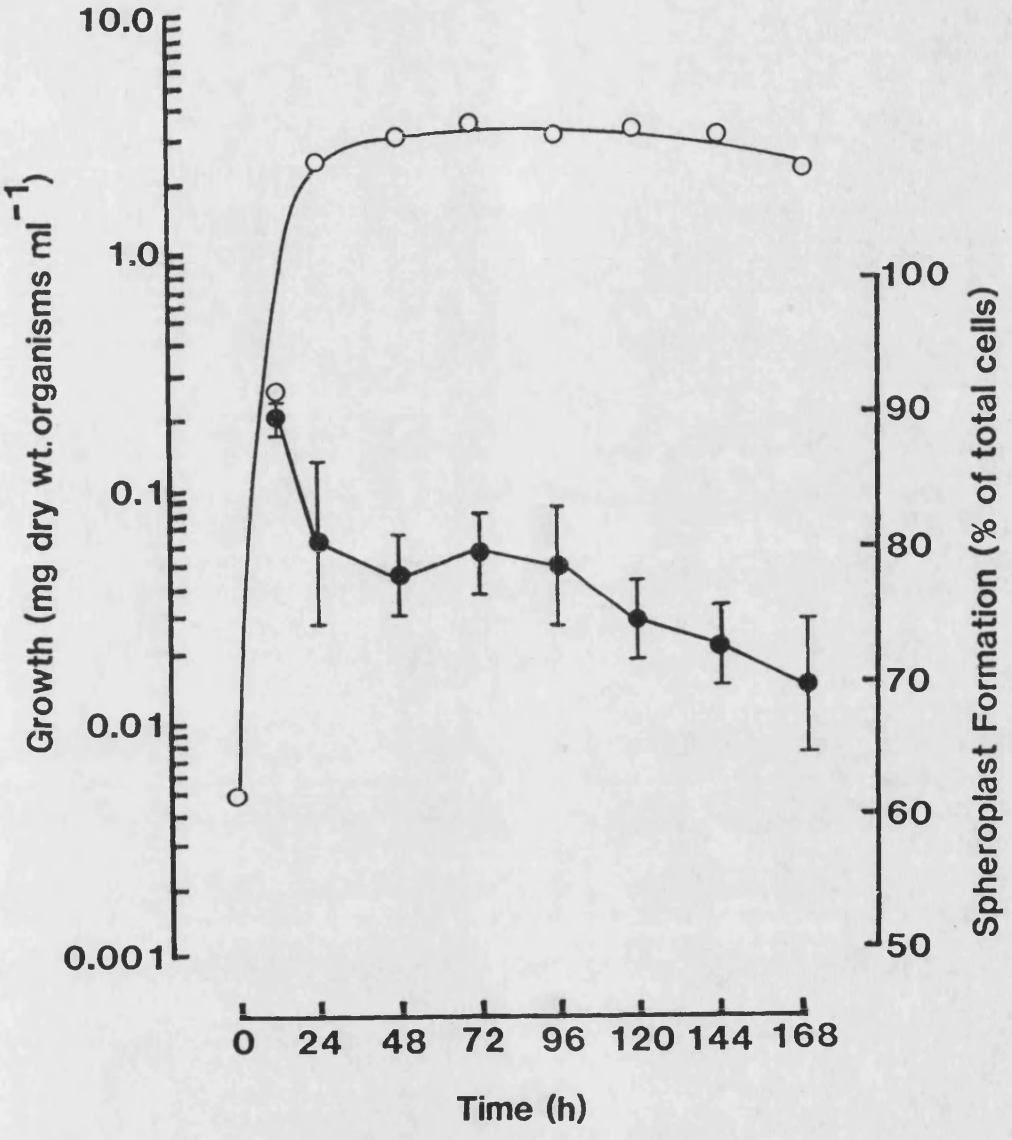



**FIGURE 13.** Time-course of self-induced anaerobic growth (○) of *Candida albicans* and the formation of spheroplasts (●) from this yeast. Values quoted for spheroplast formation are the means of three separate determinations ± SD.

Lysis of spheroplasts occurred in 1.0 and 1.2 M sorbitol giving a recovery of approximately 40%. Buffered sorbitol with a molarity of 1.5 gave increased recovery of spheroplasts, but 1.8 M buffered sorbitol gave a better recovery of 70%, although there was some shrinkage of spheroplasts in sorbitol of this molarity. Recovery of cells from 2.0 M sorbitol was no higher than from 1.8 M buffered sorbitol and severe shrinkage occurred at this concentration. In subsequent experiments requiring the use of spheroplasts, 1.8 M buffered sorbitol was used giving a recovery of approximately 70%.

## Differential Centrifugation of Cell-Free Extracts of *Candida albicans*

Cells of *C. albicans* grown for 168 h in a medium containing ($l^{-1}$) 100 g glucose and 0.5 g ammonium sulphate were disrupted by two methods, namely disintegration with a Braun homogeniser or formation and lysis of spheroplasts. The cell-free extracts from both procedures were subjected to differential centrifugation and the fractions obtained analysed for protein content, marker enzyme activity (Figs. 15 and 17) and long-chain alcohol content (Figs. 16 and 18). The data for marker enzymes are presented as relative specific activities, that is percentage of total recovered activity x percentage $^{-1}$ of total recovered protein.

## Characterisation of Fractions Obtained Following Disintegration of Cells by a Braun Homogeniser

Marker enzymes assayed in these fractions showed a fairly broad distribution of activities (Fig. 15). Over 80% of the activity of vanadate-sensitive ATPase, a marker for yeast plasma membranes, was found in P3, P12 and P25 fractions with

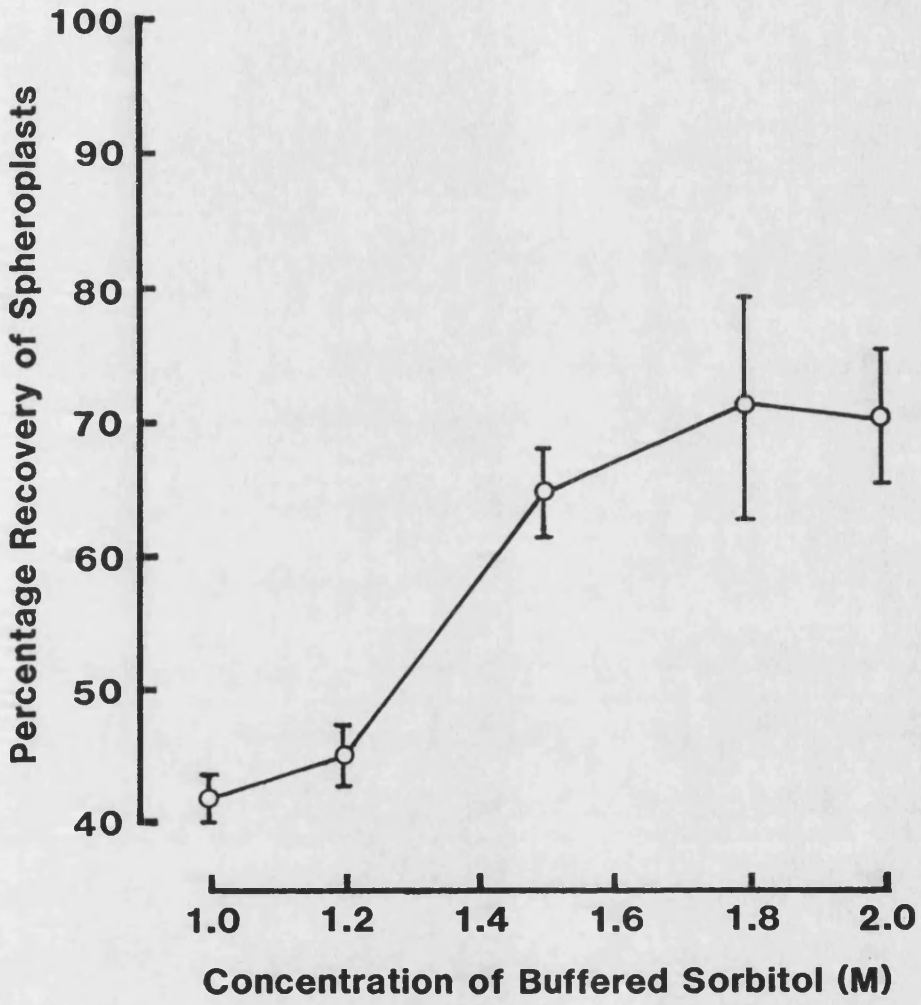



**FIGURE 14.** Stability of spheroplasts of *Candida albicans* in buffered sorbitol of various concentrations. Values quoted are the means of three separate determinations ± SD.

maximum activity located in the P25 fraction. Activity was low in the P100 fraction and very low in the S100 fraction.

The relative specific activity of the NADPH cytochrome *c* oxidoreductase in fractions obtained following ballistic homogenisation of cells showed an even distribution throughout the fractions although a peak of activity was found in the P100 pellet. This enzyme is a microsomal marker.

The peak of alkaline phosphatase activity was located in the S100 fraction representing over 45% of the total. The P3 fraction also contained a smaller peak of activity. This enzyme is a vacuolar marker in yeast.

Long-chain alcohol contents of each fraction were determined and calculated in terms of the protein content of each fraction. The results are presented as percentage of total long-chain alcohol in each fraction (Fig. 16). The distribution of tetradecanol, hexadecanol and octadecanol through the various fraction was similar. The pellets representing the P3, P12 and P25 fractions contained the major portion of these alcohols. The P100 pellet contained a smaller quantity of long-chain alcohols, but the S100 or soluble fraction contained only a very small percentage of the total long-chain alcohol.

**Characterisation of Fractions Obtained Following Disintegration of Cells by the Formation and Lysis of Spheroplasts**

Fractions obtained by this method showed a similar distribution of vanadate-sensitive ATPase and NADPH cytochrome *c* oxidoreductase to those fractions obtained following mechanical disintegration, although peak activities were more distinct and distribution less broad (Fig. 17). Over 45% of the ATPase activity

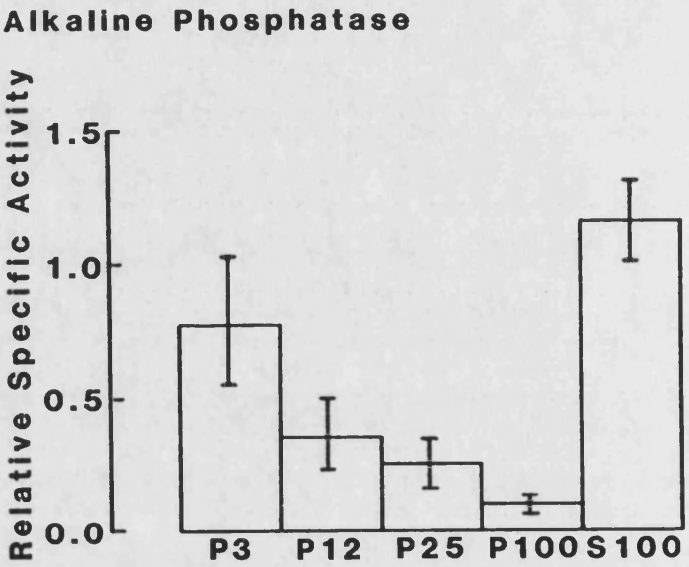
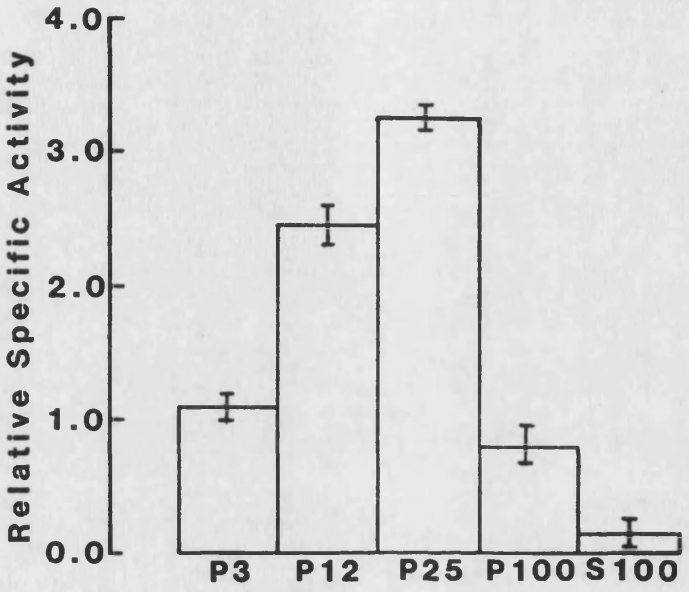
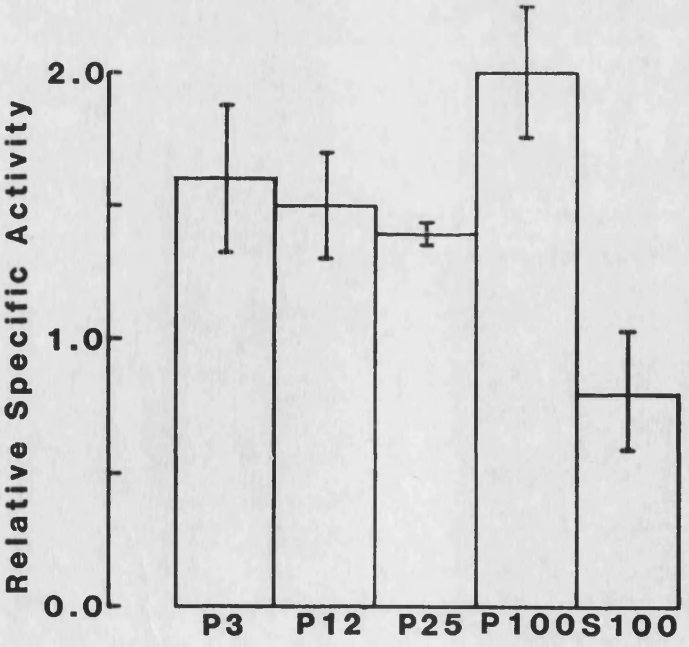



**FIGURE 15.** Relative specific activities of plasma membrane ATPase, NADPH cytochtome *c* oxidoreductase and alkaline phosphatase in various fractions obtained by differential centrifugation of a Braun homogenate of *Candida albicans* grown for 168 h. Values quoted are the means of three separate determinations ± SD.

ATPase




NADPH Cytochrome c Oxidoreductase



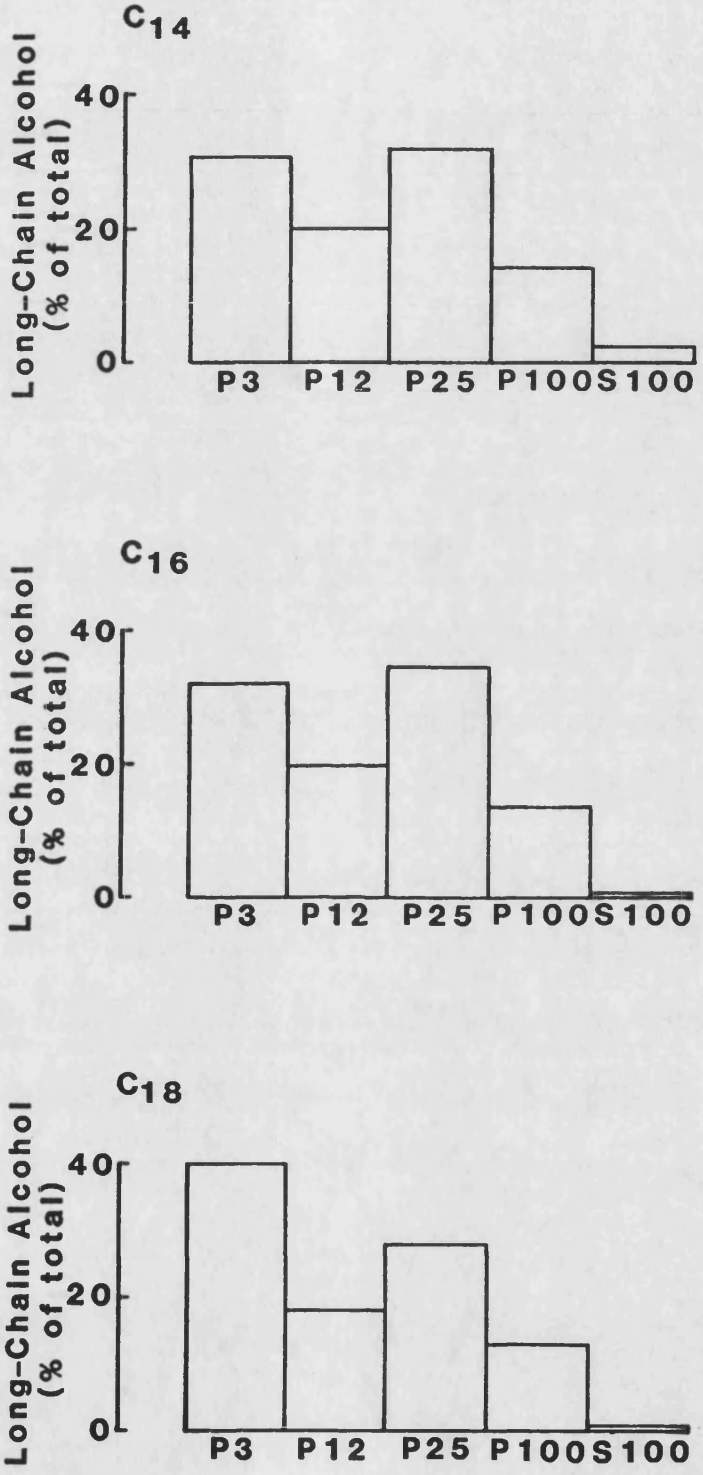
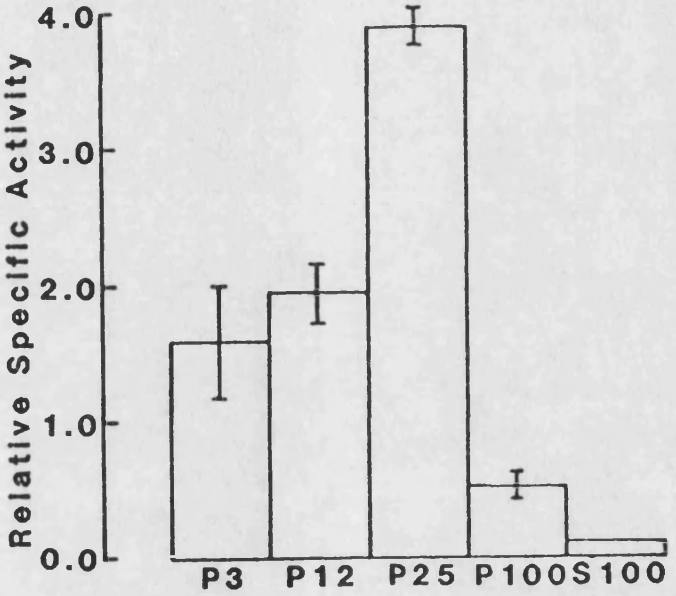


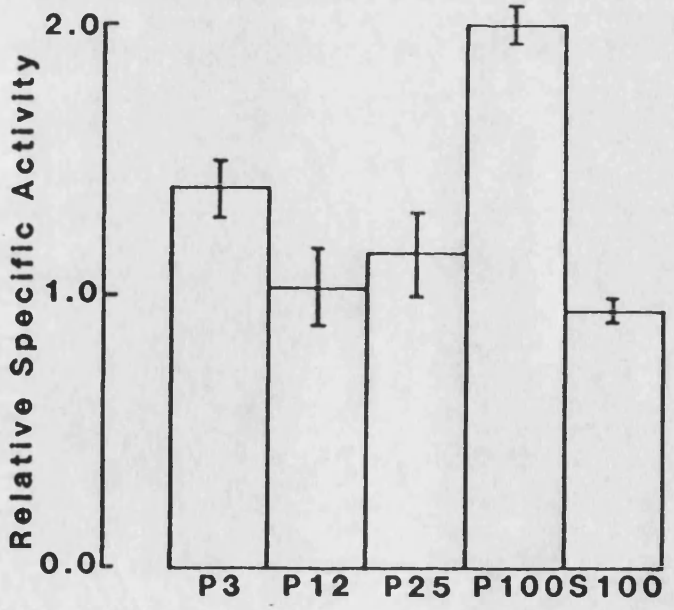

**FIGURE 16.** The percentage of $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ long-chain alcohols found in various fractions obtained by differential centrifugation of a Braun homogenate of *Candida albicans* grown for 168 h. Values quoted are of a typical experiment.

**FIGURE 17.** Relative specific activities of plasma-membrane ATPase and NADPH cytochrome *c* oxidoreductase in various fractions obtained by differential centrifugation of a spheroplast lysate of *Candida albicans* grown for 168 h. Values quoted are the means of three separate determinations ± SD.

ATPase




NADPH Cytochrome c Oxidoreductase



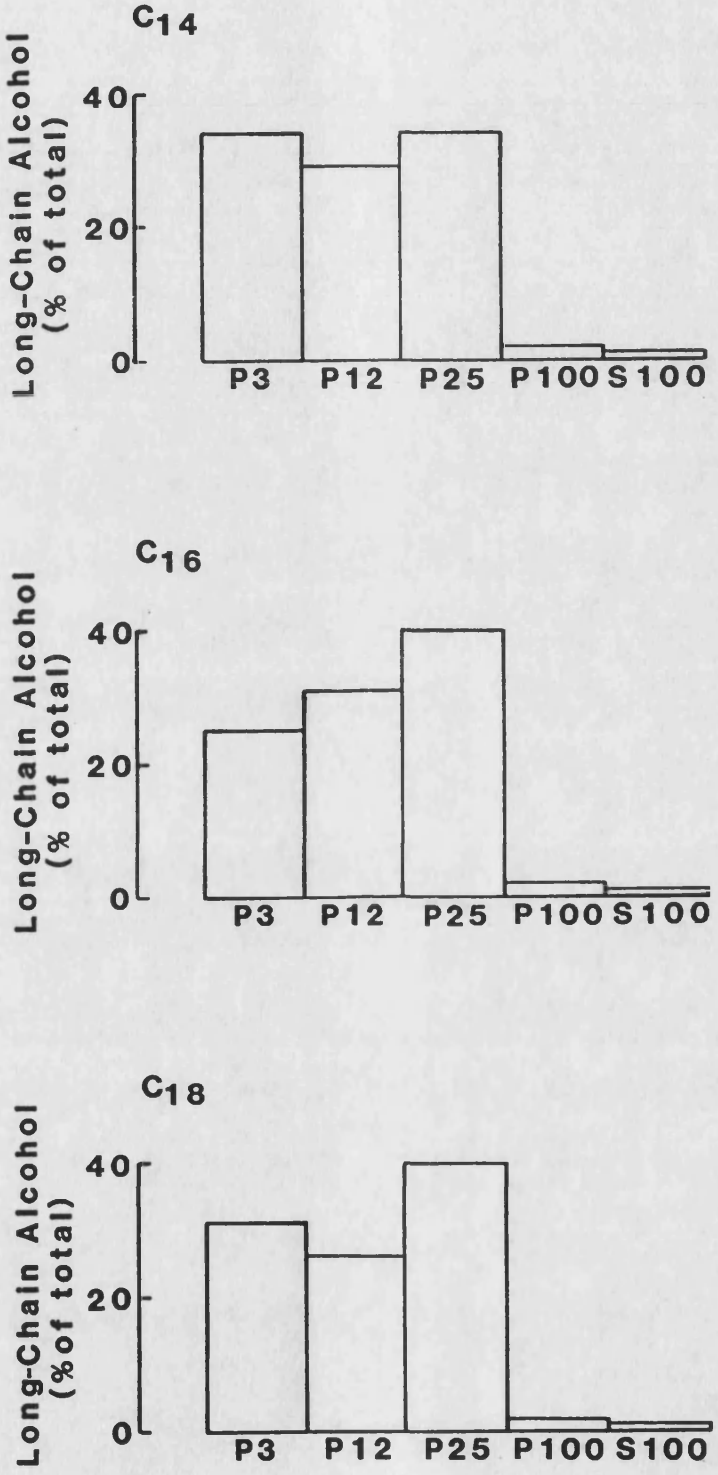



**FIGURE 18.** The percentage of $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$ long-chain alcohols found in various fractions obtained by differential centrifugation of a spheroplast lysate of *Candida albicans* grown for 168 h. Values quoted are of a typical experiment.

was located in the P25 fraction and activities in P3, P12 and P25 together represented over 85% of the total. The peak of NADPH cytochrome *c* activity was found in the P100 fraction although there was activity in all other fractions. Alkaline phosphatase was not assayed in fractions obtained following spheroplast lysis. Cytochrome P-450 was not detected in any fraction.

Long-chain alcohol contents of fractions obtained by this method had a similar distribution to the contents of fractions obtained following mechanical disintegration (Fig. 18). Maximum contents were found in P3, P12 and P25; contents in P100 and S100 were even lower than those of fractions obtained following Braun homogenisation.

**Long-Chain Alcohol Content of Cell Walls**

The homogenate resulting from the mechanical disruption of cells or the lysis of spheroplasts was subjected to a low-speed centrifugation to remove whole cells and cell walls. The supernatant resulting from this centrifugation was considered to be the cell-free extract. Lipid analysis of the pellet of whole cells and cell walls showed the presence of long-chain alcohols. The possibility therefore existed that the cell wall was the subcellular location of long-chain alcohols. Cell walls were isolated and purified by numerous washing steps and analysed for long-chain alcohols content. Table 6 shows that purified cell-wall preparations from *C. albicans* contained only very small amounts of long-chain alcohols.

**Table 6**

Long-chain alcohol contents of cell-wall preparations of *Candida albicans* grown for 168 h. Values quoted are the means of three separate determinations ± SD.

| | Long-chain Alcohol Content (ug (g dry wt organisms)$^{-1}$) | | | |
|---|---|---|---|---|
| | $C_{14:0}$ | $C_{16:0}$ | $C_{18:0}$ | Total |
| Cell walls | 13.6 ± 3.7 | 17.7 ± 6.7 | 13.3 ± 3.8 | 44.6 ± 14.0 |

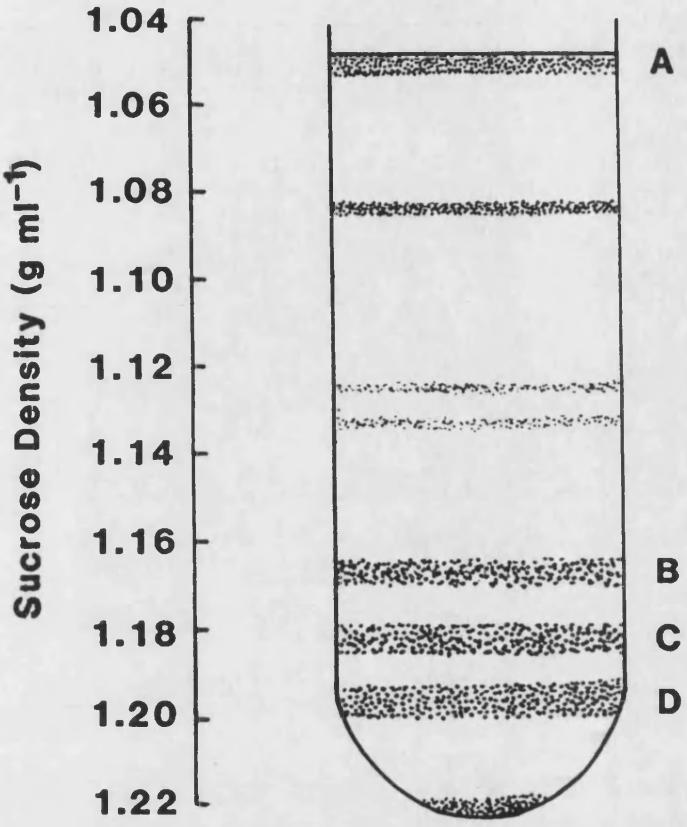



**FIGURE 19.** Drawing of a discontinuous sucrose-density gradient showing separation of cellular organelles from a spheroplast lysate of *Candida albicans* grown under self-induced anaerobic conditions. A, B, C and D represent major bands.

## Fractionation of Spheroplast Lysates of *Candida albicans* on Sucrose-Density Gradients

Spheroplast lysates from self-induced anaerobically-grown *C. albicans* produced two distinct regions of turbidity in the discontinuous sucrose-density gradient (Fig. 19). A single, milky-white band at the top of the gradient (1.05 g $ml^{-1}$) represented the low-density vesicle fraction. A series of three closely associated bands were found at sucrose densities between 1.16 g $ml^{-1}$ and 1.20 g $ml^{-1}$. A small yellow pellet of whole cells was located at the bottom of the gradient (Fig. 19).

When spheroplast lysates of *C. albicans* grown for 72 h were fractionated on sucrose-density gradients,tetradecanol, hexadecanol and octadecanol were located in fractions containing high vanadate-sensitive ATPase activity (1.16 to 1.20 g $ml^{-1}$). Tetradecanol was also located in almost equivalent amounts in the low-density vesicle fraction (1.05 g $ml^{-1}$) which had a very low ATPase activity (Fig. 20).

Long-chain alcohols from spheroplast lysates of *C. albicans* grown for 120 h were also located in fractions containing high ATPase activity (Fig. 21). The low-density vesicle fraction (1.05 g $ml^{-1}$) of these lysates contained tetradecanol, hexadecanol and octadecanol with an especially high content of tetradecanol. The fractions from these lysates were further characterised by analysis of the protein content and the microsomal enzyme marker, NADPH cytochrome *c* oxidoreductase. The peaks for protein content were found to correspond to densities of 1.06g $ml^{-1}$ and 1.20 g $ml^{-1}$. The NADPH cytochrome *c* oxidoreductase activity was found to peak at a density of 1.08 g $ml^{-1}$.

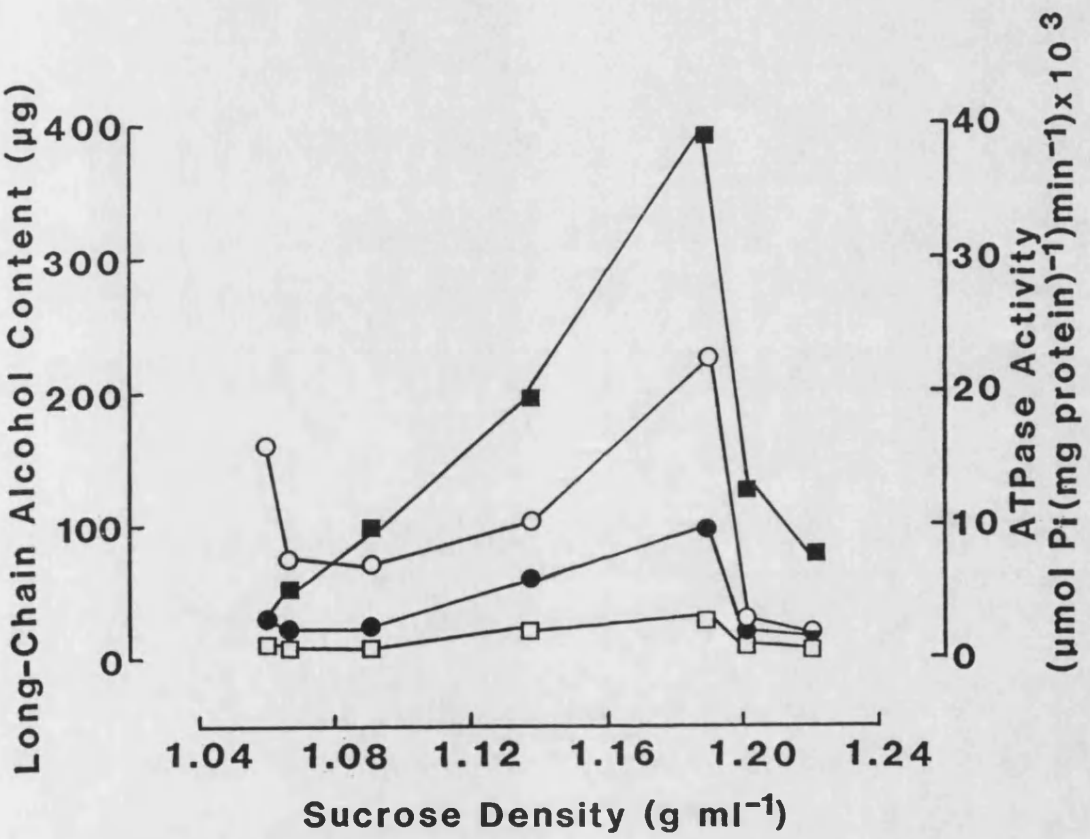
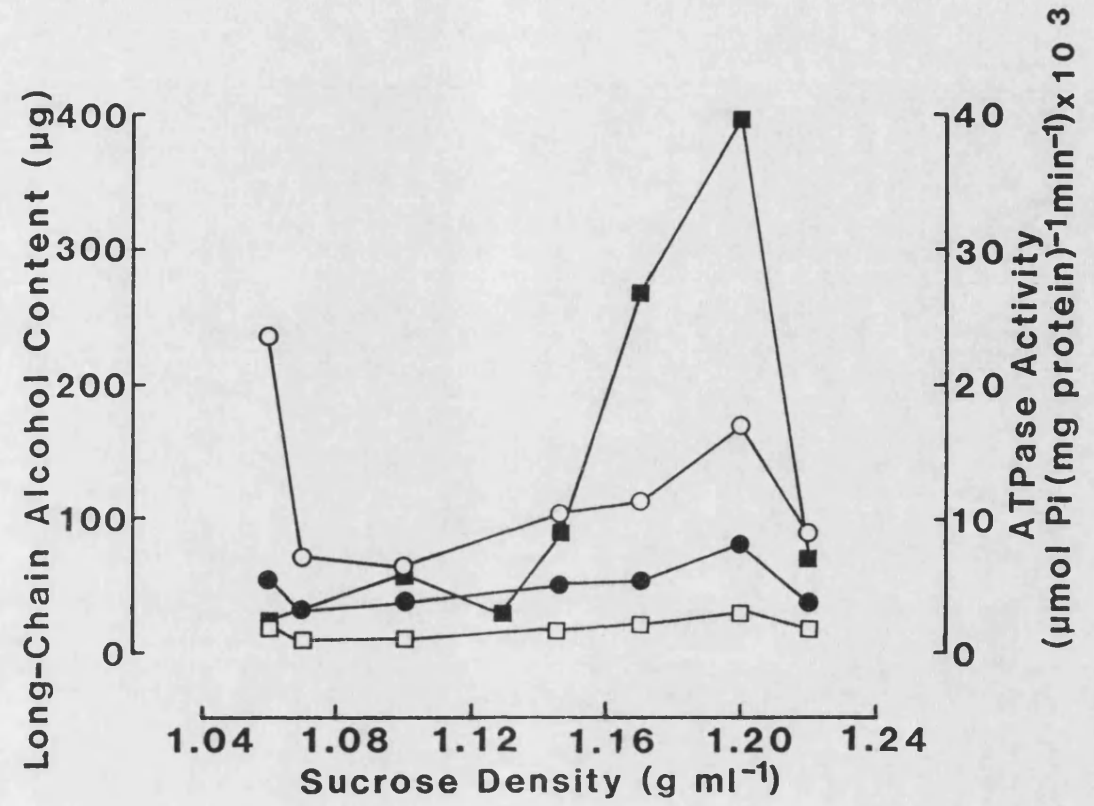



**FIGURE 20.** Distribution of $C_{14:0}$ (○), $C_{16:0}$ (●) and $C_{18:0}$ (□) long-chain alcohols and plasma-membrane ATPase (■) activity through a fractionated spheroplast lysate of *Candida albicans* grown under self-induced anaerobic conditions for 72 h. Values quoted are of a typical experiment.

**FIGURE 21.** Distribution of $C_{14:0}$ (○), $C_{16:0}$ (●) and $C_{18:0}$ (□) long-chain alcohols, plasma-membrane ATPase activity (■), protein content (△) and NADPH cytochrome *c* oxidoreductase activity (▲) through a fractionated spheroplast lysate of *Candida albicans* grown under self-induced anaerobic conditions for 120 h. Values quoted are of a typical experiment.


Long-Chain Alcohol Content (µg)
400
300
200
100
0
40
30
20
10
0
ATPase Activity
(µmol Pi (mg protein)$^{-1}$min$^{-1}$)x10 3
1.04
1.08
1.12
1.16
1.20
1.24
Sucrose Density (g ml$^{-1}$)

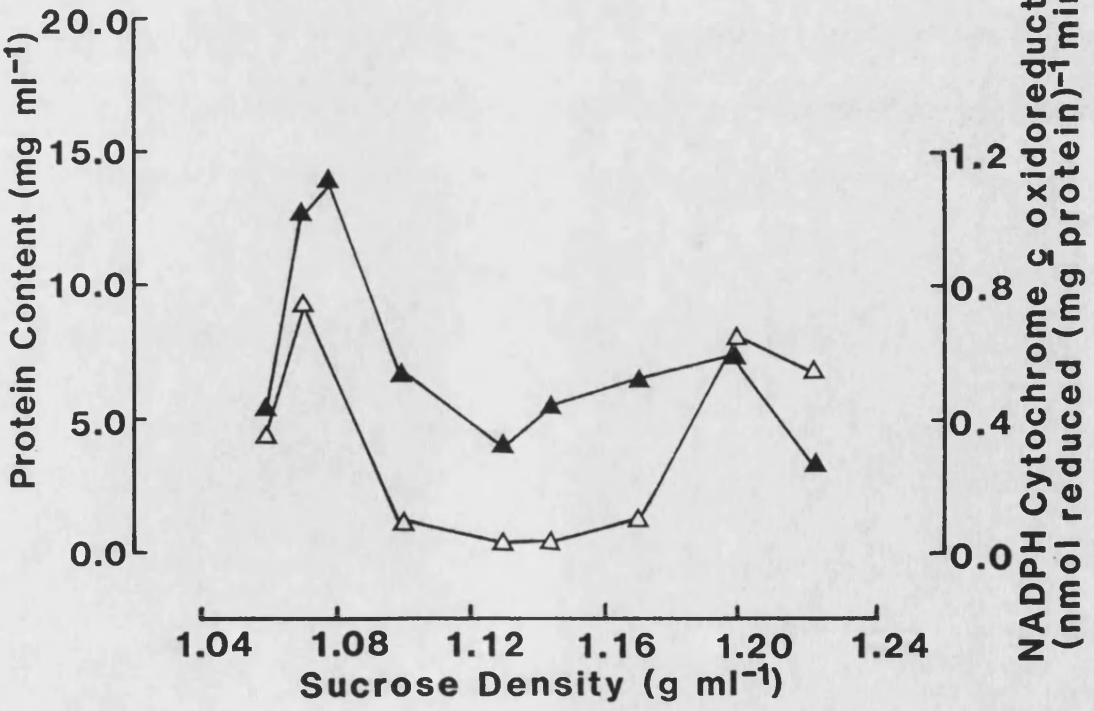
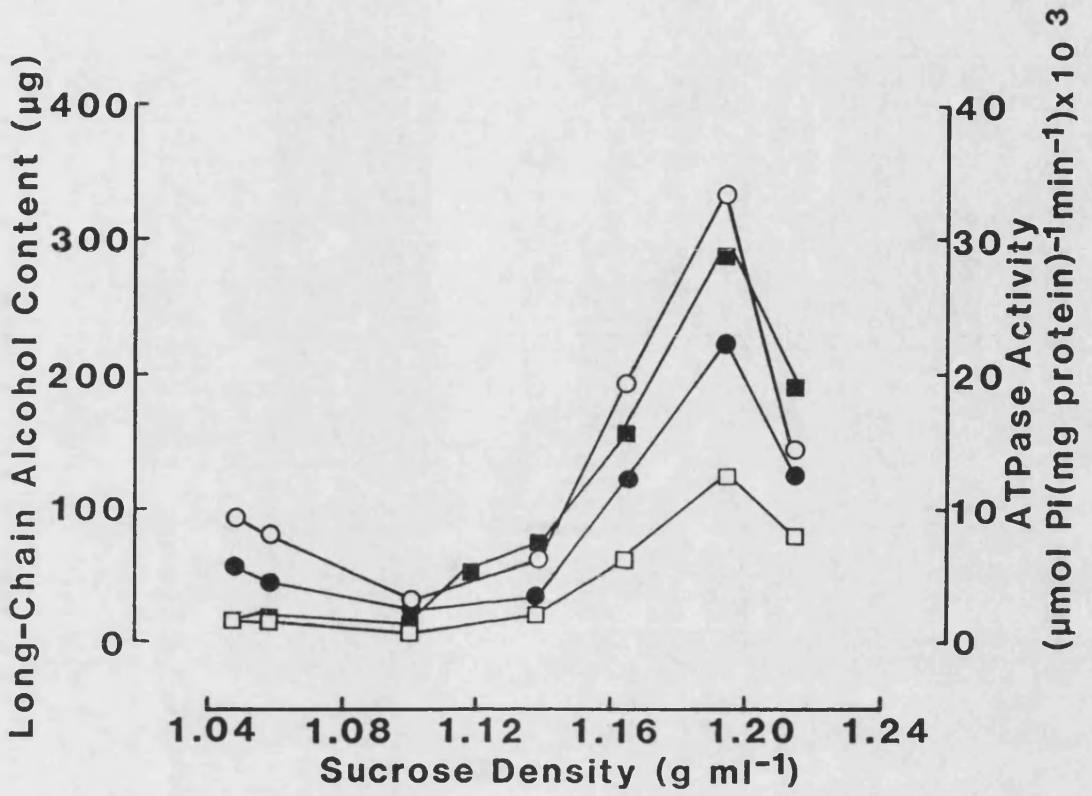
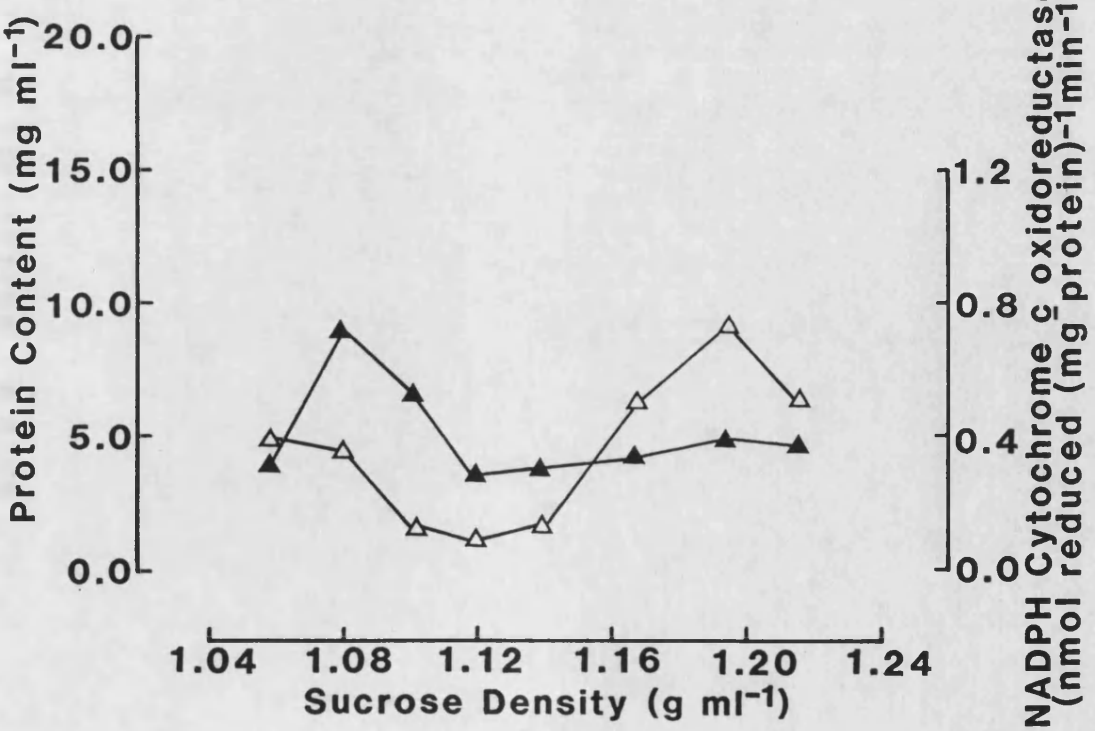

Protein Content (mg ml$^{-1}$)
20.0
15.0
10.0
5.0
0.0
1.2
0.8
0.4
0.0
NADPH Cytochrome c oxidoreductase
(nmol reduced (mg protein)$^{-1}$ min$^{-1}$)
1.04
1.08
1.12
1.16
1.20
1.24
Sucrose Density (g ml$^{-1}$)

**FIGURE 22.** Distribution of $C_{14:0}$ (○), $C_{16:0}$ (●) and $C_{18:0}$ (□) long-chain alcohols, plasma-membrane ATPase activity (■), protein content (△) and NADPH cytochrome *c* oxidoreductase activity (▲) through a fractionated spheroplast lysate of *Candida albicans* grown under self-induced anaerobic conditions for 168 h. Values quoted are of a typical experiment.


Long-Chain Alcohol Content (µg)
400
300
200
100
0
40
30
20
10
0
ATPase Activity
(µmol Pi(mg protein)-1min-1)x10 3
1.04
1.08
1.12
1.16
1.20
1.24
Sucrose Density (g ml-1)


Protein Content (mg ml-1)
20.0
15.0
10.0
5.0
0.0
1.2
0.8
0.4
0.0
NADPH Cytochrome c oxidoreductase
(nmol reduced (mg protein)-1min-1)
1.04
1.08
1.12
1.16
1.20
1.24
Sucrose Density (g ml-1)

When spheroplast lysates of *C. albicans* grown for 168 h were fractionated on sucrose-density gradients tetradecanol, hexadecanol and octadecanol were once again located in fractions containing high vanadate-sensitive ATPase activity (1.16 to 1.19 g ml $^{-1}$). All three long-chain alcohols were also located in the low-density vesicle fraction located at a density of 1.05 g ml$^{-1}$ (Fig. 22). The protein content peaked around sucrose densities of 1.06 and 1.19 g ml$^{-1}$. The peak for NADPH cytochrome *c* oxidoreductase activity was located around 1.07 g ml$^{-1}$.

## MUTAGENESIS STUDY.

This preliminary study into the possibility of improving long-chain alcohol production of yeasts through mutation used cells of the yeast *C. tropicalis* grown aerobically for 48 h in YNB/DM in 250 ml baffled flasks. These cells were treated with nitrosoguanidine as a mutagen and estimates of petite and auxotroph frequencies indicated that the treatment was effective in producing mutations with each treatment with nitrosoguanidine (M2, M3 and M4 series; Table 7).

When the treated cells were applied to a simple sucrose-density gradient there was a major band of cells at the top of sucrose with a density of 1.22 g ml$^{-1}$ and only a very minor band at the top of sucrose with a density of 1.25 g ml$^{-1}$. After repeating three cycles of the enrichment procedure, the band at the top of 1.25 g ml$^{-1}$ became more turbid (Fig. 23). A number of individual colonies from lower bands were grown under self-induced anaerobic conditions for 168 h and analysed for long-chain alcohol production and triacylglycerol content (Table 7). Increased long-chain alcohol production was not seen in those isolates analysed, but all isolates studied showed a decrease in triacylglycerol production. The parent strain

Table 7

Frequency of petites and nutritional auxotrophs and triacylglycerol contents of high-density isolates *Candida tropicalis* following treatment with N-Methyl-N'-nitro-N-nitrosoguanidine. Each series (M2, M3 and M4) represents a separate treatment with the mutagen. nd indicates triacylglycerol was not detected.

| Isolate | Petite Frequency (%)* | Auxotroph Frequency (%) | Triacylglycerol Content (% cell dry wt) |
|---|---|---|---|
| M2 Series | 29.5 | 1.8 | |
| M2/1 | | | nd |
| M2/2 | | | 0.78 |
| M2/3 | | | 0.99 |
| M2/4 | | | 0.33 |
| M2/5 | | | 0.42 |
| M3 Series | 21.7 | 4.3 | |
| M3/1 | | | 0.84 |
| M3/2 | | | 1.21 |
| M3/3 | | | 0.48 |
| M3/4 | | | 0.79 |
| M3/5 | | | 0.75 |
| M4 Series | 45.1 | 0.9 | |
| M4/1 | | | nd |
| M4/2 | | | 0.34 |
| M4/3 | | | 0.88 |
| M4/4 | | | nd |
| M4/5 | | | 0.89 |

*Petite frequencies estimated on colony size.

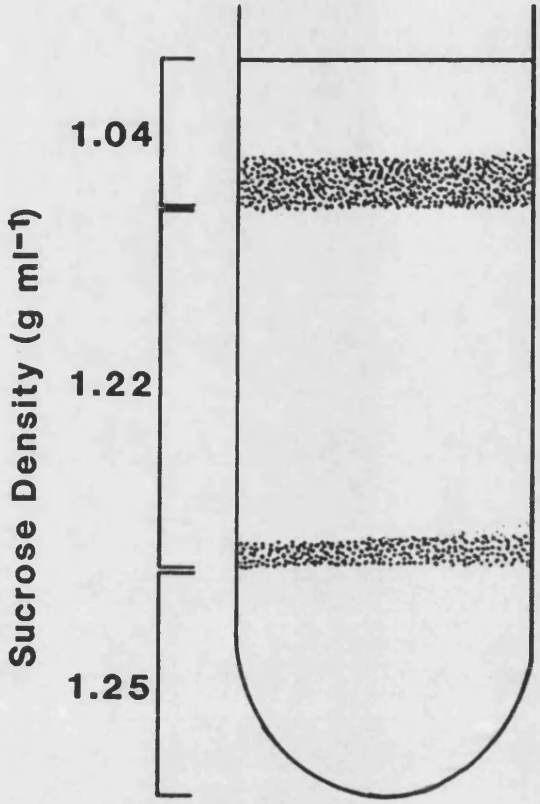



**FIGURE 23.** Drawing of a discontinuous sucrose-density gradient showing separation of low- and high- buoyancy cells of *Candida tropicalis* grown under aerobic conditions in medium containing 0.5 g ammonium sulphate $l^{-1}$.

of *C. tropicalis* grown under these conditions had a triacylglycerol content of approximately 15% cell dry wt. whereas the low-buoyancy isolates from the 1.25 g $ml^{-1}$ band showed a triacylglycerol content ranging from 0.33 to 1.21% cell dry wt. This indicates perturbation of the triacylglycerol synthesis pathway.

# DISCUSSION

This project is an extension of a study carried out by White (1987) in the Zymology Laboratory at the University of Bath. This earlier work was the first extensive study of long-chain alcohol biosynthesis in yeasts and involved the survey of fourteen strains of yeasts for fatty-alcohol content. The best producer in this survey was a strain of *Candida albicans* and the time-course of biosynthesis indicated that long-chain alcohols are secondary metabolites in this yeast. Production of long-chain alcohols was shown to be strongly influenced by oxygen availability and also by amount and type of carbon source available to the yeast.

Long-chain alcohols are not commonly reported as components of microbial lipid extracts. There have been some reports of long-chain alcohols in bacteria (Fixter and Fewson, 1974; Lloyd and Russell, 1983a), but reports of their occurrence in yeasts are rare . Muratov *et al.* (1979) reported the presence of wax esters in *Candida guilliermondii* grown on *n*-alkanes. The major alcohols were saturated with chain lengths of $C_{10}$, $C_{16}$ and $C_{17}$. Long-chain alcohols were also reported in *C. tropicalis* grown on glucose or hexadecane (Davidova *et al.*, 1978). The fatty alcohols were present as components of wax esters and were found in higher amounts when the growth substrate was hexadecane. A patent has been filed recently to cover the production of wax esters by *Apiotrichum curvatum* (Sekula, 1988). However, this yeast produces wax esters only when fattened on fatty alcohols and no wax esters were detected in extracts from organisms grown in fatty alcohol-free medium.

A number of workers have reported the presence of "unidentified" lipid fractions from lipid extracts of yeast. Watanabe and Takakuwa (1984) separated the lipids

extracted from glucose-grown *Sacch. rouxii* and found an "unidentifiable" spot between free sterols and free fatty acids. An "unidentifiable" spot in this same position was also found by Zalashko *et al.* (1979) on analysis of lipid extracts of *Rh. glutinis* grown on glucose. Both of these studies utilized similar TLC solvent systems to that employed in the present study. It is possible that these lipids are long-chain alcohols. Alternatively they may be members of the highly unsaturated isoprenoid alcohol series which have similar mobilities to long-chain alcohols when separated by TLC.

Long-chain alcohols are products of some importance to the detergent and chemical industries and there is a commercial interest in producing these chemicals from a microbial source. White *et al.* (1988) found long-chain alcohols in amounts approaching 1 mg (g dry wt. organisms)$^{-1}$ in *C. albicans*. However, this yeast is not the most suitable industrial organism due to its opportunistic pathogenicity. Thus the first objective of this project was to extend the studies of these earlier workers to include other yeasts in the hope of finding an alternative, non-pathogenic organism capable of producing significant amounts of long-chain alcohols.

Analysis of extracts of *C. albicans* grown for 168 h confirmed the values obtained by White *et al.* (1987, 1988). A number of other yeasts were surveyed for long-chain alcohol production. *Candida* sp. 107 is an oleaginous yeast (Gill *et al.*, 1977) and under conditions of glucose-excess and nitrogen-deficiency accumulates lipids largely as triacylglycerols. Under the conditions used in this study, *Candida* sp. 107 produced long-chain alcohols in reasonable amounts. Maximum long-chain alcohol contents were encountered in stationary-phase cultures, a pattern suggesting secondary metabolism. This accumulation was shown to be transient with maximum long-chain alcohol content at 120 h followed by a decline in content. However, production in this yeast was found to

be somewhat variable and the large amount of triacylglycerol produced by *Candida* sp. 107 caused a number of experimental problems associated with extraction of relatively small amounts of fatty alcohol. Hence production of long-chain alcohols by this yeast was not pursued further.

A number of other yeasts were also assessed for long-chain alcohol production, but amounts detected were small. *Candida utilis* contained larger amounts than the other yeasts assessed, but even these amounts were several-fold less than the amounts of long-chain alcohols detected in *C. albicans*. Further studies on long-chain alcohols were confined to this latter yeast, except for the research on long-chain alcohol producing mutants of *C. tropicalis*.

The research carried out by White *et al.* (1987) suggested that long-chain alcohols produced by *C. albicans* were secondary metabolites. This was confirmed and extended in the present study. Synthesis of long-chain alcohols in this yeast is delayed until the stationary-phase of growth and appears to be subject to a complex regulatory mechanism.

Long-chain alcohol production is favoured by conditions of excess carbon source and a deficiency of a nitrogen source. These are also the conditions favouring accumulation of triacylglycerol in oleaginous yeasts (Rattray *et al.*, 1975). The biochemical explanation for lipid accumulation in these yeasts proposed by Botham and Ratledge (1979) involved regulation of several biosynthetic enzymes by levels of ATP and NADPH. These workers suggested that glucose uptake may continue unchecked into cells when the nitrogen source limits growth and subsequent metabolism of glucose leads to excess energy equivalents, generated by respiration, and NADPH arising from the pentose-phosphate pathway. The build up in ATP and depletion of AMP affects a number of enzymes including

ATP:citrate lyase leading to a build up of acetyl-CoA which becomes available for fatty acid biosynthesis (Botham and Ratledge, 1979).

The carbon:nitrogen ratio in the medium appears to be a regulatory factor in the production of a number of secondary metabolites, such as the phenolic compound trihydroxytoluene (THT) produced by *Aspergillus fumigatus* (Ward and Paxter, 1974). A study of fatty-acid synthesis and THT production in this organism showed that fatty-acid biosynthesis occurred throughout the exponential phase of growth and continued into the stationary phase whereas THT synthesis occurred only at a late stage in the incubation period and commenced only after the exhaustion of ammonium ions from the culture medium. Addition of an ammonium salt completely inhibited THT production without affecting either the growth rate or pH value of the medium. Both fatty-acid and THT biosynthesis utilize the same thioester substrates and depend upon the presence of a synthetase complex of enzymes. Activity of the fatty-acid synthetase was found to be diminished during the stationary-phase of growth and THT production was suggested as a means of maintaining CoA levels within the cell. However, the authors indicated that the regulation is likely to be more complex than a simple diversion of substrates from fatty-acid biosynthesis (Ward and Paxter, 1974).

Cephalosporin production by *Streptomyces clavuligerus* is also subject to nitrogen catabolite regulation (Aharonowitz and Demain, 1979). Growth and antibiotic production were supported by various amino acids, including asparagine and glutamine. Ammonium salts supported a somewhat slower growth rate, and antibiotic production was very poor in the presence of these inorganic nitrogen sources. The negative effect of excess ammonium ions was also observed when ammonia was present in addition to asparagine. Under these media conditions there was increased mycelial mass, but this was accompanied by a decrease of about 75% in cephalosporin production. This effect was seen when $NH_4Cl$ was

added at 24 h or earlier, before onset of antibiotic synthesis and thus could not be attributed to a specific inhibition of antibiotic-forming enzymes. When $NH_4Cl$ was added 48 h into the fermentation, there was essentially no effect on cephalosporin synthesis. These data indicated that the biosynthetic machinery responsible for cephalosporin production is regulated by nitrogen metabolism and is established during the trophophase before antibiotics can be detected in the medium. Repression of formation of antibiotic synthetases by ammonium ions appears to be a possible regulatory mechanism; however, this remains to be conclusively proven.

Complex nitrogen sources are found to be best for production of many secondary metabolites (Drew and Wallis, 1983) and it appears to be the rate of utilization of a nitrogen source and not its identity which most directly relates to the regulatory mechanism. However, to date there is very little information on nitrogen catabolite regulation in yeast (Wiame *et al.*, 1985).

Regulation of long-chain alcohol production by *C. albicans* appears to be subject to a complex control mechanism. White *et al.* (1988) established that high concentrations of carbon source favoured long-chain alcohol production and results from the present study show that amounts are further elevated if ammonium sulphate is present in limiting concentrations. These conditions favour a build up of ATP, NADPH and acetyl-CoA which, in oleaginous yeasts, result in excessive production of triacylglycerol. *Candida albicans* is not classified as an oleaginous yeast and reported contents of triacylglycerols in this yeast are extremely low (Ratledge and Evans, 1988). The possibility exists that acyl-CoA residues in *C. albicans* are diverted into long-chain alcohol synthesis thereby releasing CoA and thus helping to maintain a stable internal environment under adverse conditions.

This oversimplification belies the complex regulatory mechanisms involved in secondary metabolism control. However, fatty-alcohol biosynthesis can be seen to have a number of features in common with that of several other secondary metabolites. Secondary metabolism and growth compete for key intermediates of primary metabolism. Catabolite repression during growth overrides induction of the enzymes involved in secondary metabolism; however nutritional limitations remove this repression (Malik, 1980). Under conditions of nitrogen-deficiency, or deficiency of another nutrient other than the carbon source, growth is limited and primary metabolites and intermediates accumulate. Elevated concentrations of these metabolites are the precursors and inducers of pathways leading to secondary metabolites. Under these conditions, ATP tends to accumulate and in fact high levels of ATP, NADPH, glutamate and 2-oxoglutarate provide powerful driving forces for ammonia assimilation (Neijssel and Tempest, 1979). Accumulation of ATP and NADPH has been inferred from formation of glycogen in micro-organisms (Tempest *et al.*, 1967). High levels of ATP inhibit citrate synthase and other enzymes that metabolize products of glucose metabolism such as acetyl-CoA (Malik, 1980). Removal of growth-associated regulation of secondary metabolism pathways may channel acetyl-CoA towards synthesis of fatty acids, polyketides or, in the case of *C. albicans*, towards fatty alcohols.

## SUBCELLULAR LOCATION OF LONG-CHAIN ALCOHOLS IN *Candida albicans*

The subcellular location of long-chain alcohols in *C. albicans* is of interest not least because it may help to attribute a possible role for these secondary metabolites within the cell. This is the first study of the subcellular location of these alcohols in yeasts, and it was necessary to obtain some general indication of possible subcellular location before purification of any fraction was attempted.

This was achieved using an analytical fractionation technique, based on differential centrifugation, modified from Delaissé *et al.* (1981). Initial experiments employed cells disrupted by Braun homogenisation and then subjected to a differential centrifugation regime. Marker enzyme studies showed that the S100 and P100 fractions of the Braun homogenate of *C. albicans* essentially represented the soluble and microsomal fractions, respectively. Absence of large amounts of long-chain alcohols from these two fractions indicated that the long-chain alcohols were largely found in the fractions obtained at lower centrifugation speeds.

In all fractionation experiments, the cell homogenate obtained after cell disruption was subject to a very low-speed centrifugation step to remove whole cells, cell walls and large debris. The supernatant resulting from this pellet was considered to represent the cell-free extract. However, analysis of the pellet showed considerable fatty-alcohol content and the possibility existed that the cell wall itself was the location for long-chain alcohols. However, the small amounts of long-chain alcohols detected in purified preparations of cell walls of *C. albicans* indicate that this organelle is not the principal subcellular location of fatty alcohols.

Disruption of cells using a Braun homogeniser is an extremely efficient means of cell breakage. However, the redistribution of cell components and constituents using this method is a potential problem, a factor illustrated by the broad distribution of marker enzymes and long-chain alcohols. Clearly, any attempt to fractionate membranous elements of yeast cells must start with gentle disruption of the cells in physiological medium (Dubé *et al.*, 1973) and to achieve this a method was developed to obtain spheroplasts from cells of *C. albicans* in the stationary-phase of growth. Previous workers have achieved spheroplast formation from this yeast in the past (e.g. Sugawara, 1966), but the cells used in

these studies were harvested in the exponential-phase of growth. Brown (1970) found *C. albicans* to be resistant to the action of lytic enzymes. Susceptibility to wall degradation diminished gradually during the stationary-phase of growth and it was postulated that such alterations in susceptibility were due to changes in the cell-wall composition concomitant with depletion of metabolites in the medium. In order to avoid prolonged incubation of *C. albicans* with the cell-wall degrading enzymes, cells were pretreated with mercaptoethanol and EDTA. Thiol agents reduce disulphide cell-wall mannoproteins in *Sacch. cerevisae* (DeNobel *et al.*, 1989) and this pretreatment was effective in allowing formation of spheroplasts from stationary-phase cultures of *C. albicans*.

Differential centrifugation of spheroplast lysates re-affirmed the conclusions arising from the differential centrifugation of ballistically-disrupted cells. The microsomal and cytosolic fractions, as identified by marker enzymes, contained only a very small proportion of the fatty alcohols. Long-chain alcohols were detected in the three fractions arising from the lower speed centrifugations of the cell extract, with a peak in the fraction obtained following centrifugation at 25 000*g*. The vanadate-sensitive ATPase, a marker for plasma membrane (Willsky, 1979), had a similar distribution hence the next logical step was to isolate and purify a plasma-membrane fraction.

There are a limited number of reports of yeast plasma-membrane purifications in the literature. Garcia-Mendoza and Villanueva (1967), working with a spheroplast lysate of *C. utilis*, used a fraction sedimenting between 1 500 and 15 000*g* as their plasma-membrane fraction. Nurminen *et al.* (1976) disrupted cells of *Sacch. cerevisiae* using glass beads and subjected the homogenate to zonal centrifugation in Ficoll or sucrose. The fractions obtained were analysed for marker enzyme, phospholipid and sterol contents. Schibeci *et al.* (1973) also fractionated spheroplast lysates of *Sacch. cerevisiae* and used plasma membrane-

specific radioactive labels to follow the distribution of membrane upon fractionation. Isolation and chemical characterisation of plasma membranes of *C. albicans* (Marriott, 1975a, b) involved osmotic lysis of spheroplasts formed from this yeast followed by fractionation using a combination of differential and discontinuous sucrose-density gradient flotation centrifugations. This research was mainly concerned with differences in protein, lipid and carbohydrate contents between yeast and mycelial forms of this yeast (Marriott, 1975a) although the work was extended to include the enzymic activity of purified plasma membranes (Marriott, 1975b). Ouabain-sensitive, $Mg^{2+}$-dependent ATPase and mannan synthetase were both found to have a subcellular location in the plasma membrane.

In this present study, sucrose-density gradients were used to fractionate spheroplast lysates of *C. albicans*. When lysates of cells grown for 168 h were fractionated by this method, the peak of long-chain alcohol content coincided with the peak of vanadate-sensitive ATPase. The microsomal marker NADPH cytochrome *c* oxidoreductase showed a peak elsewhere in the density gradient. The peak of vanadate-sensitive ATPase activity and long-chain alcohol content also coincided when the cells were harvested at 72 h or 120 h before fractionation on sucrose-density gradients. This indicates that the principal subcellular location of long-chain alcohols in this yeast is probably the plasma membrane. The reproducibility of this finding lends credence to this surmise, although confirmation of this conclusion could be gained from electron microscopy of fractions.

Due to the lipid nature of long-chain alcohols, a membrane location is not unexpected, although the many essential functions of the plasma membrane do not immediately suggest this membrane as an ideal site for a potentially disruptive molecule. However, long-chain alcohol production is delayed until the stationary-

phase of growth and, at this stage of the growth cycle, the membrane may be able to withstand a greater degree of disruption than during the phase of exponential growth.

The long-chain alcohols of the wax ester component of *M. cryophilus* were found primarily in the cell envelope by Lloyd and Russell (1983b) with a five-fold difference between the inner membrane and the outer membrane, unlike phospholipids which showed an uneven distribution of classes between the two membranes. Growth temperature has been shown to influence the degree of unsaturation of wax esters of this bacterium (Russell, 1978) and it has been suggested that wax esters may function as modulators of membrane fluidity (Russell and Volkman, 1980). However, nitroxide-labelled fatty acid spin probes incorporated into membrane lipids were unaffected by the presence of wax esters and these workers were uncertain as to the exact function of wax esters in the membrane (Lloyd and Russell, 1983b, 1984).

Long-chain alcohols, assumedly derived from wax esters have also been reported in the outer membranes of an *Acinetobacter* sp. (Thorne *et al.*, 1973) and have been assumed to serve as structural components of bacterial membranes (Bryn *et al.*, 1977). However, Fixter *et al.* (1986) found that accumulated waxes in nitrogen-limited bacteria were degraded to water-soluble molecules and $CO_2$ during carbon starvation. These workers suggested that wax esters served as energy-reserve materials in these bacteria. It was argued that, as carbon-limited bacteria showed a ratio of moles of wax esters to g atoms of lipid phosphorus of about 0.04 and nitrogen-limited bacteria of 1.6, this change could not be reconciled with a role in membrane fluidity (Fixter *et al.*, 1986).

Most studies of secondary metabolites have concentrated on commercially-important aspects of the biosynthesis of various industrially-significant products.

These aspects include means of maximising product accumulation and ease of recovery of products from the cultivation broth. As a consequence very few studies have been made on the subcellular location of secondary metabolites, although the accumulation site and transport of antibiotics have been studied in high- and low-yielding mutants of antibiotic-producing streptomycetes (Kurylowicz, 1977). Electron microscopy showed that the surface of the mycelium of the actinomycetes was smooth in the early hours of fermentation, but substructures were found on the surface of high-yielding strains later in the fermentation. The surface of low-yielding strains remained smooth throughout. Ultra-thin sections of cells of the high-yielding actinomycetes showed an electron-dense cell wall and, in older hyphae, numerous invaginations of the cell membrane. Few of these membranous structures were seen in low-yielding strains. These workers suggested a role for these invaginations in the membrane in production and accumulation of antibiotics in actinomycetes. These organisms appear to be able to tolerate high concentrations of antibiotics within the membrane. However, these antibiotics are peptides, tetraenes or macrolides and thus resemble long-chain alcohols only by virtue of being secondary metabolites.

Fractionation of spheroplast lysates of *C. albicans* showed that a certain proportion of long-chain alcohols were located in the low-density vesicle fraction. This proportion changed throughout the time-course of growth. Cells harvested after 72 h had smaller amounts of long-chain alcohols in the vesicle fraction whereas cells harvested after 120 h contained larger amounts in this fraction. Vesicles may be involved in the transport of long-chain alcohols from their site of synthesis to their site of accumulation. This role has been assigned for vesicles in the transport and secretion of exocellular enzymes by yeasts (Herrero *et al.*, 1980) and transport of proteins in *Sacch. cerevisiae* (Scheckman, 1982). A role for low-density vesicles in envelope growth has also been postulated in *Sacch. cerevisiae* (Henschke *et al.*, 1983).

When cells of *C. albicans* in the stationary-phase of growth with high long-chain alcohol contents were transferred from nitrogen-limited medium into fresh medium containing ammonium ions there was a lag phase of some 12 h. This lag phase did not occur in transferred cultures grown under conditions encouraging low long-chain alcohol content. Ballman and Chaffin (1979) transferred stationary-phase cells of *C. albicans* into fresh medium and observed increased cell numbers after 3 h. The present study showed a rapid decline in long-chain alcohol content during the lag phase, with a minimum value after 16 h. A similar pattern was seen in the triacylglycerol content of *Sacch. cerevisiae* when stationary-phase cells were used as an inoculum (Taylor and Parks, 1979). The amount of triacylglycerol decreased by 50% during the first three generations of growth while the amount of phospholipid was increasing. These workers suggested that triacylglycerol accumulated as a result of curtailment of phospholipid synthesis in the late exponential growth phase and was then utilized for phospholipid synthesis when growth resumed. Further evidence for this was gained from pulse-chase experiments which showed a loss of radioactive label from the triacylglycerol fraction and an equal gain in the phospholipid fraction upon regrowth of stationary-phase cells (Taylor and Parks, 1979).

Analysis of cell-free medium following the transfer of stationary-phase *C. albicans* showed an increase in long-chain alcohol content. The content in the medium was found to be approximately equivalent to loss from the cells. The percentage of viable cells did not fall during this time; thus presence of long-chain alcohols in the medium could not be attributed to lysis of dead cells.The increase in long-chain alcohol content of the medium occurred mainly during the first hours following transfer of cells whilst the cells were in lag phase. These results are significant when considering the role of long-chain alcohols as secondary metabolites within the cell. Stationary-phase cells appear to be able to accommodate a certain amount of long-chain alcohols apparently in the plasma

membrane, although the reason for production of these molecules is unknown. When the cultural conditions change and the medium is no longer growth-limiting, it would seem that growth is unable to occur until the long-chain alcohol content of the cell is of a sufficiently low value. Loss of long-chain alcohols from the cell begins within a few hours of transfer and the cells remain in the lag phase of growth for several hours. Resynthesis of long-chain alcohols occurs once organisms have reached the stationary-phase of growth.

Further evidence that the cells must dispose of long-chain alcohols before growth can occur is indicated by double transfer experiments. A second transfer, 48 h after the first transfer, was again accompanied by a rapid decline in long-chain alcohol content. The lowest content was approximately the same value as the lowest content following the first transfer, indicating a critical level of long-chain alcohols that the organism can accommodate whilst growing.

There is recent evidence that a long-chain alcohol oxidase is present in a number of yeasts (Hommel and Ratledge, 1990; Kemp *et al.*, 1988). A fatty alcohol is the first product of alkane oxidation and an inducible long-chain alcohol oxidase has been proposed to catalyse the step from fatty alcohol to aldehyde (Kemp *et al.*, 1988) although the accepted mechanism appears to be the dehydrogenation of the alcohol by a long-chain specific NADH-dependent dehydrogenase (Gallo *et al.*, 1973). The fatty oxidase activity studied in *C. bombicola* is involved in glycolipid production and appears to be constitutive with high activities whether the cells are grown on *n*-alkanes or carbohydrates (Hommel and Ratledge, 1990). However, neither oxidation nor dehydrogenation appear to be important routes for the removal of long-chain alcohols from *C. albicans* upon re-initiation of growth. The fact that fatty alcohols are excreted into the medium rather than metabolized is another indication that it is the pathway of production of these metabolites

rather than the alcohols themselves that plays a metabolic role in maintaining a stable internal environment within the organism.

These data indicate that accumulation of alcohols in the plasma membrane represents a self-induced inhibitory phenomenon. Assuming that all the fatty alcohols are located plasma membrane and that this membrane in *C. albicans* contains 55% protein and 45% lipid, and accounts for 10% of the dry wt. of organisms (Henschke and Rose, 1990) then the plasma membrane in organisms from 168-h cultures probably contains between 7 and 10% of its dry wt. in the form of long-chain alcohols. Removal of long-chain alcohols from the cell is presumably to allow plasma membrane proteins and enzymes to carry out their growth-associated functions. Enzymes and transport proteins in the plasma membrane containing 7 - 10% of its lipid as long-chain alcohols may not have the required annuli of essential phospholipids and sterols (Prasad and Rose, 1986). The plasma membrane probably does not easily accommodate tetradecanol molecules because of their short chain length, and it is significant that these alcohols are preferentially excreted following re-inoculation. Moreover, a plasma membrane containing long-chain saturated alcohols would have a lower fluidity than one free from such alcohols. Release of long-chain alcohols from organisms could be a process for restoring membrane fluidity to levels required for activity of plasma-membrane proteins and hence growth.

It is of interest to note that as the cellular content of long-chain alcohols increases due to a decrease in nitrogenous nutrient availability, the relative proportion of tetradecanol increases. This may be the earliest step in the synthesis of fatty-acyl residues that acyl-CoA esters can be diverted into long-chain alcohol synthesis. Future work could include a study of the ability of cell-free extracts to convert dodecanoyl-CoA and other shorter chain esters into long-chain alcohols.

The decrease in long-chain alcohol content following re-inoculation of *C. albicans* into fresh medium shows a disproportionately large decrease in tetradecanol content before the re-initiation of growth. This may be an indication that this shorter-chain alcohol is more disruptive to plasma-membrane function than either hexadecanol or octadecanol.

## REDUCTION OF PALMITOYL-CoA USING CELL-FREE EXTRACTS OF *Candida albicans*

Work by White *et al.* (1988) suggested that long-chain alcohol biosynthesis in *C. albicans* occurred via reduction of CoA esters of fatty acids. Evidence supporting this view was the *in vivo* reduction of exogenously-supplied odd-chain fatty acids to odd-chain fatty alcohols. Lipid extracts of non-supplemented *C. albicans* did not contain odd-chain fatty alcohols. White *et al.* (1988) confirmed this reductive route by demonstrating palmitoyl-CoA reduction to hexadecanol by cell-free extracts. However, this demonstration was very preliminary and the work presented here is, to my knowledge, the most extensive study of the biosynthesis of long-chain alcohols by yeast yet reported.

Production of long-chain alcohols via a reductive route has been demonstrated in a range of organisms including mammals (Natarajan and Schmid, 1975; Bishop and Hajra, 1981), plants (Khan and Kolattukudy, 1975; Kolattukudy, 1971) and bacteria (Day and Goldfine, 1978; Wang *et al.*, 1972). In all cases the fatty acid is activated through formation of an acyl-CoA derivative before reduction to the corresponding alcohol, probably via a fatty aldehyde intermediate. This two-step reduction is likely to be catalysed by two separate, though closely associated enzymes. In *C. albicans*, the subcellular location of the reductase activity was found to be microsomal. This is also the site of synthesis in rat brain (Bishop and

Hajra, 1981), sparrow uropygial gland (Kolattukudy and Rogers , 1978), *Euglena gracilis* (Kolattukudy, 1970) and *Mycobacterium tuberculosis* (Wang *et al.*, 1972). However, the reductase activity is found in the soluble fraction of extracts of bovine cardiac muscle (Kawalek and Gilbertson, 1976), broccoli leaves (Kolattukudy, 1971) and *Clostridium butyricum* (Day *et al.*, 1970). This result contrasts with White *et al.* (1988) who found acyl-CoA reductase activity in both the microsomal and soluble fractions, with greater activity in the soluble fraction. This discrepancy may be due to the method employed to disrupt the yeast cells. Cell-disintegration using a Braun homogeniser is an extremely efficient means of cell breakage but may cause solubilization of microsomal enzymes.

In general, the cofactor requirement for microsomal acyl-CoA reductases is for NADPH (Kolattukudy and Rogers, 1978; Snyder and Malone, 1970; Wang *et al.*, 1972) and this was also the cofactor required by the enzyme system in this current study, although NADH also allowed some reductase activity. This also contrasts with the data obtained by White *et al.* (1988) which showed that NADPH and NADH were equally effective as cofactors for this system. Non-specificity for nicotinamide adenine dinucleotide molecules is unusual although it has been noted in the alkane oxidation system of *C. rugosa* (Boulton and Ratledge, 1984). White *et al.* (1988) suggested that a transhydrogenase capable of interconverting NADH and NADPH may have been present in the crude enzyme preparation.

A comparison of methods of microsomal precipitation showed that centrifugal precipitation resulted in higher reductase activity than precipitation using $CaCl_2$. Addition of $CaCl_2$ to a reaction mixture containing a centrifugally precipitated microsomal preparation caused a drop in activity indicating a possible inhibitory effect of this compound. Inhibition of acyl-CoA activity by $CaCl_2$ has also been noted by Bishop and Hajra (1981). These workers noted a drop in activity of 50% in the presence of 5 mM $CaCl_2$.

In this system the maximum rate of conversion of palmitoyl-CoA to hexadecanol was 0.12 nmol (mg protein)$^{-1}$ h$^{-1}$. This is comparable to the rate of acyl-CoA reductase activity in rat brain microsomes (Natarajan and Sastry, 1976) and *M. tuberculosis* (Wang *et al.*, 1972) but is considerably lower than the activity in *E. gracilis* (Kolattukudy, 1970b) and preputial gland tumours (Synder and Malone, 1970). Levels of fatty alcohols found in these latter two tissues are considerably higher than those found in *C. albicans* so that higher rates of activity are to be expected. Analysis of the *in vivo* rate of hexadecanol formation in *C. albicans* shows a maximum rate of 4.8 µg (g dry wt.)$^{-1}$ h$^{-1}$. Assuming that protein constitutes approximately 40% of the cell dry mass, the *in vitro* rate of 0.12 nmol (mg protein )$^{-1}$ h$^{-1}$ is approximately equivalent to 11.6 µg (g dry wt. )$^{-1}$ h$^{-1}$. Thus the reported *in vitro* activity is sufficient to account for the *in vivo* rate of hexadecanol production. However, this must be treated with caution because of unknown parameters of the internal environment.

The data reported in this study show that a large excess of substrate is required for maximum reductase activity. This phenomenon has also been encountered by a number of other workers (e.g. Bishop and Hajra, 1981; Wang *et al.*, 1972) and there are two possible reasons for this effect. The presence of radiolabelled free fatty acid in the reaction products is indicative of an acyl-CoA hydrolase in the crude enzyme preparation. The preferred substrate for the hydrolase is acyl-CoA in the form of micelles and inclusion of bovine serum albumin in the incubation system helps to break up the micelles (Bishop and Hajra, 1981). However, some hydrolysis of the acyl-CoA is unavoidable with an unpurified enzyme preparation. In addition to this, detergent properties of palmitoyl-CoA lead to a surface absorption effect so that a large fraction of the substrate is unavailable to the enzyme (Kolattukudy and Rogers, 1978). Considering the particulate nature of the enzyme, the detergent effect of the substrate and the role played by bovine

serum albumin in this assay, the apparent $K_m$ value calculated for reductase activity should be taken with the usual precautions.

No attempt was made in the present study to purify the reductase(s) and indeed there has been only one successful purification of a microsomal acyl-CoA reductase to date (Khan and Kolatttukudy, 1975). The aldehyde intermediate was not detected amongst the reactions products in this present study. This intermediate is generally only detected in small amounts in microsomal systems (Kolattukudy, 1970b) and requires the use of aldehyde-trapping agents.

The change in palmitoyl-CoA reductase activity of *C. albicans* over the time-course of hexadecanol production showed that a peak of activity coincided with the maximum rate of hexadecanol formation. The fall in reductase activity seemed to slightly preceed the fall in the rate of alcohol production and this pattern of enzyme activity would seem to indicate that the enzyme or enzymes involved are produced in excess at the beginning of the phase of long-chain alcohol production. There have been few studies of the enzyme activity over the time-course of production of a secondary metabolite. However, production of gramicidin S synthetases by *Bacillus brevis* has been extensively studied by Matteo *et al.* (1975, 1976). In batch culture, the pattern of enzyme activity was similar to palmitoyl-CoA reductase activity in *C. albicans* with gramicidin synthetase activities appearing for only a short time and disappearing rapidly. These workers suggested that the set of conditions favouring synthetase appearance exist for only a short time in the batch-culture growth cycle and that there was an active removal of enzyme activity (Matteo *et al.*, 1975). Enzyme activity was also studied at different growth-rates during continuous culture (Matteo *et al.*,1976) and it was concluded that growth rate, rather than a specific nutrient limitation, regulated the level of synthetases in the chemostat. The results

suggested that neither a high growth rate nor a very low growth rate were compatible with gramicidin synthetase activity.

## SEARCH FOR LONG-CHAIN ALCOHOL-PRODUCING MUTANTS.

An increase in productivity of a biosynthetic process can be achieved by a number of means including control of physical parameters such as agitation or incubation temperature, addition to the growth medium of a precursor or metabolic poison and by manipulation of nutritional conditions (Perlman, 1973). However, although product yield may be improved by optimizing the cultural conditions, productivity is ultimately under the control of the genome. A wild-type organism may only produce limited quantities of a secondary metabolite even under optimal cultural conditions. Thus a major increase in productivity can be achieved through the modification of the organism's genome using either mutation or recombination techniques.

Although recombination techniques are proving useful in many areas of biotechnology, the lack of basic genetic information about industrial organisms means that most strain-development programmes are based on mutation (Williams and Franklin, 1980) and this technique has played an important role in the development of penicillin and cephalosporin antibiotics. Standard mutation techniques cause inheritable changes in a large number of genes and it is not possible to determine which genes will be affected. It is, therefore, necessary to differentiate the few superior producers from the many inferior producers using an appropriate selection procedure. The isolation of mutants overproducing secondary metabolites is difficult because these products are not required for growth and there is also a lack of knowledge about the control of biosynthetic pathways leading to secondary metabolites. Many strain-improvement

programmes have been based on empirical screening (Elander, 1979) although the use of selection techniques is becoming increasingly wide-spread (e.g. Casey *et al.*, 1990; Hill *et al.*,1986).

The mutagenesis study carried out as part of this current work was a preliminary investigation into the feasibility of using mutation techniques to improve the long-chain alcohol yield of a low-producing strain. *Candida tropicalis* was chosen for two reasons. Firstly, long-chain alcohols were detected in this yeast, although amounts were small; secondly, the level of triacylglycerol indicated an active fatty acid biosynthetic pathway, which is probably important for precursor supply for long-chain alcohol synthesis. The mutagen employed was nitrosoguanidine which appears to cause error-prone duplication through damage to DNA polymerase III (Sermonti, 1978) and is useful because of its ability to induce high mutation frequencies at high survival rates using simple protocols. The selection procedure, designed to isolate mutants with lower buoyancies than the wild-type, was based on the theory that these isolates may have disrupted triacylglycerol biosynthetic pathways. The possibility existed that excess fatty-acyl residues resulting from this disruption may be diverted into long-chain alcohol synthesis. However, although the selection procedure was effective in selecting low-triacylglycerol producers, none of the isolates analysed had elevated long-chain alcohol contents. This was a preliminary study and the disruption in triacylglycerol synthesis is an encouraging result. A wider survey or an additional selection procedure may reveal a mutant of *C. tropicalis* defective in triacylglycerol synthesis with a high content of long-chain alcohols.

The reason for long-chain alcohol synthesis in yeasts remains obscure and as yet this class of lipids has not been assigned any specific metabolic role in these organisms. The significance of some other microbial secondary matabolites is also unknown although specific roles have been postulated for others. Neijssel and Tempest (1979) suggested that some secondary metabolite production may be the result of overflow metabolism as a consequence of nutrient limitation. The authors suggest that a metabolic compromise occurs in order to circumvent bottle-necks imposed by a specific growth-limitation. This allows growth to continue, although at a diminished rate, and prevents intermediary metabolites accumulating to toxic levels. A similar theory, first proposed by Bu'lock (1961), suggests that secondary metabolism maintains synthetic processes in operational order during times of metabolic stress and avoids metabolic problems arising from the accumulation of large concentrations of low molecular-weight compounds.

As suggested earlier, the presence of long-chain alcohols in *C. albicans* may be due to diversion of fatty-acyl residues to a reductive route. Free fatty acids have been shown to be toxic to yeasts (Gershon and Shanks, 1979; Gill and Ratledge, 1972; Hünkova and Fencl, 1977) and long-chain alcohol formation may avoid this toxic effect while maintaining a stable internal environment through avoidance of the accumulation of low molecular-weight intermediates, such as acetyl-CoA, and energy equivalents. Alternatively, long-chain alcohols may simply be the result of a futile metabolic pathway induced by metabolic conditions. Detection of long-chain alcohols amongst the metabolic products of yeasts is another addition to the diversity of lipids that micro-organisms are known to synthesize and successfully accommodate and is a reflection of the flexibility of yeasts allowing them to adapt to changing external environments.

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